THE ROLE OF 5-HT_{2C}RECEPTOR DENSITY ON BEHAVIOUR IN MICE

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Declaration

I hereby declare that this thesis has been composed entirely by myself and that the work presented here is the result of my own independent investigation, except where otherwise acknowledged in the text.

No part of this work has not been submitted for any other degree or professional qualification.

Paula Stevenson

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Abstract

The neurotransmitters serotonin (5-HT) and dopamine (DA) play roles in eating disorders, mood disorders, such as depression and anxiety, and in the regulation of locomotion. The 5-HT_{2C} receptor is one of fourteen 5-HT receptor subtypes that is expressed in regions of the brain including the hippocampus, amygdala, dorsal striatum, nucleus accumbens (NA) and substantia nigra, and is therefore implicated in behaviours and disorders associated with these regions. 5-HT has been shown to exert both a tonic and phasic inhibitory control, through the 5-HT_{2C} receptor, on the firing rate and bursting activity of DA-containing neurones in the ventral tegmental area which enhances DA release in the NA and prefrontal cortex. In addition, the 5-HT_{2C} receptor is under the control of a monophasic diurnal rhythm and is in a position to alter circadian regulation and behaviour due to its expression in the suprachiasmatic nucleus (the light entrainable oscillator (LEO)).

It was hypothesised that elevating expression of the 5-HT_{2C} receptor would have a detrimental effect on mood and cause hypolocomotion while reducing 5-HT_{2C} receptor expression would improve mood, cause hyperphagia, obesity and hyperlocomotion. In order to investigate these hypotheses mouse models that either over- or under-expressed the 5-HT_{2C} receptor were implemented. The 5-HT_{2C} receptor expression pattern and levels were confirmed in all mouse lines. A behavioural phenotype of hypolocomotion and increased anxiety in the 5-HT_{2C} receptor over-expressing mice and hyperphagia, obesity and hyperlocomotion in the 5-HT_{2C} receptor under-expressing mice were found the latter is conistent with current literature. During backcrossing of the mouse lines onto the C57Bl/6 genetic background the abnormal behavioural phenotypes were lost suggesting that 5-HT_{2C} receptor function is particulary sensitive to the genetic background on which it is being expressed. In response to altered expression levels of 5-HT_{2C} receptor, compensatory alterations were found in the 5-HT system, with an inverse relationship existing between both the 5-HT_{1A} receptor mRNA expression levels and 5-HT release in the hippocampus with the expression levels of the 5-HT_{2C} receptor. Over-expression of 5-HT_{2C} receptor appears to inhibit DA release in the cortex. The circadian experiments showed that under-expressing the 5-HT_{2C} receptor did not

alter the regulation of the food entrainable oscillator and there was a suggestion that the regulation of the LEO was affected.

In summary, these results demonstrate that altered expression of 5-HT2C receptors results in abnormal behaviours consistent with its role in psychiatric disorders, but that the outcome is dependent on the genetic background.

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List of Abbreviations

3-MT	3-Metoxytryptamine
5-HIAA	5-Hydroxyindoleacetic Acid
5-HT	5-Hydroxytryptamine (Serotonin)
5-HTP	5-Hydroxy-L-Tryptophan
5-HTT	5-HT Transporter
AAADC	Aromatic Amino Acid Decarboxylase
ACTH	Adrenocorticotropic Hormone
ADAR	Adenosine Deaminase Acting on RNA
AGS	Sound Induced Convulsions
BBB	Blood-Brain Barrier
BLA	Basolateral Amygdala
COMT	Catechol-O-Methyl-Transferase
CNS	Central Nervous System
CPu	Caudate Putamen
DA	Dopamine
DAG	Diaglycerol
DAT	DA Transporter
DDC	Amino Acid Decarboxylase
DG	Dentate Gyrus
DNA	Deoxyribonucleic Acid
DOPAC	Dihydroxyphenyl-Acetic Acid
FEO	Food Entrainable Oscillator
GPRCs	G-Protein Coupled Receptors
HPA	Hypothalamic-Pituitary Adrenal

HVA	Homovanillic Acid
IP_3	Inositol Phosphate
KO	Knock-out
L-DOPA	L-Dihydroxyphenylalanine
LEO	Light Entrainable Oscillator
MAO	Monoamine Oxidase
mCPP	1-(m-Chlorophyl)-Piperazine
Min (s)	Minute(s)
mRNA	Messenger RNA
NA	Noradrenaline
OCD	Obsessive Compulsive Disorder
PI	Phosphatidylinositol
PIP2	Phosphatidylinositol (4,5)-bis-phosphate
PMAT	Plasma Membrane Monoamine Transporter
REM	Rapid Eye Movement
RHT	Retinohypothalamic Tract
RNA	Ribonucleic Acid
SAD	Seasonal Affective Disorder
SCN	Suprachiasmatic Nucleus
SERT	5-HT Transporter
SN	Substantia Nigra
SNRI(s)	Serotonin and Noradrenaline Reuptake Inhibitor(s)
SSRI(s)	Selective Serotonin Reuptake Inhibitor(s)
STR	Striatum
TCA	Tricyclic Antidepressants
TH	Tyrosine Hydroxylase

TPH	Tryptophan Hydroxylase
Trp	L-Tryptophan
VTA	Ventral Tegmental Area
WT	Wild Type

CHAPTER 1:

INTRODUCTION

1.1 The 5-HT system

In the central nervous system (CNS), 5-hydroxytryptamine (5-HT) is a monoaminergic neurotransmitter that due to its diffuse anatomical distribution, modulates many different aspects of brain function, and has been implicated in a variety of brain disorders. In particular it is involved in many psychiatric disorders including anxiety, depression and obsessive compulsive disorder. Maurice Rapport first isolated and named 5-HT (to indicate its origin from blood serum and its effect on vascular tone) in 1948 (Rapport *et al*., 1948) and Gaddam later discovered its presence in the CNS (Green., 2008; Murphy., 2008).

1.1.2 5-HT Synthesis

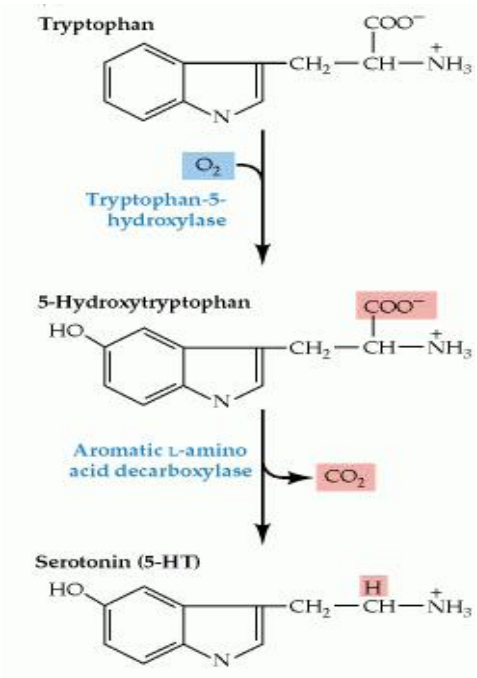
5-HT is one of the less abundant neurotransmitters in the brain and is a biogenic amine with a pK of 9.8, which means that it is charged at physiological pH and so is unable to cross the blood-brain barrier (BBB) or diffuse into cells from the extra-cellular space (Diksic & Young., 2001). For this reason 5-HT must be synthesised within neurons which are defined as 5-HT neurons due to the presence of the necessary enzymes required for 5-HT synthesis. The two-step metabolic pathway of 5-HT synthesis (see Figure 1a) requires the amino acid L-tryptophan (Trp), obtained from the diet, which is hydroxylated by tryptophan hydroxylase (TPH) into 5-hydroxy-L-tryptophan (5-HTP). 5-HTP is then converted to 5-HT by aromatic amino acid decarboxylase (AAADC). There are two TPH isoforms; TPH1 which controls 5-HT synthesis in the periphery and TPH2 which is brain specific (Walther *et al*., 2003; Zhang *et al*., 2004; Gutknecht *et al*., 2009). The TPH gene, which codes for the rate-limiting enzyme of 5-HT biosynthesis, has been cloned (Boularand *et al*., 1990) and mapped on 11p15.3-p14 (Craig *et al*., 1991). Expression of the TPH gene is under tight regulation at both the transcriptional and post-transcriptional levels (Cote *et al*., 2002). This first step requires Trp plus two additional substrates which are oxygen and tetrahydrobiopterin. Tetrahydrobiopterin is sometimes referred to as

a cofactor as it is converted to dihydrobiopterin, then reduced back to tetrahydrobiopterin and reused. TPH is not saturated with any of its substrates so increasing the level of terabhydrobiopterin (in rat: Miwa *et al*., 1985), oxygen (in rat: Davis *et al*., 1973; in dog: Diksic *et al*., 1991) or Trp increases the rate of 5-HT synthesis. Interestingly genetic polymorphisms in both TPH1 and TPH2 can influence susceptibility to anxiety and depression (Nash *et al*., 2005, Zhang *et al*., 2005). A single nucleotide polymorphism at the *mTPH2* locus (C1473G) reduced 5-HT synthesis by 55% when expressed in PC-12 cells (Zhang *et al*., 2004) and in mice this polymorphism determines differences in 5-HT synthesis rates but not tissue content in C57BL/6 mice (Siesser *et al*., 2010). Female *Tph2* KO mice have increased aggressive behaviour (Alenina *et al*., 2009) and female aggression is directly linked to anxiety and depressive disorders (Bosch *et al*., 2005). There is also evidence that the expression of TPH is affected by ovarian hormones which suggests a possible mechanism for premenstrual stress syndrome and postpartum depression (Hiroi *et al*., 2006). AADC is not a rate limiting step but does require pyridoxal phosphate as a cofactor.

Immediately following synthesis a 5-HT binding protein (SBP) protects both cystolic 5-HT and 5-HT in growth cones from monoamine oxidase (MAO) before it is stored into vesicles (Tamir & Gershon., 1979; Ivgy-May *et al*., 1994;Tamir *et al*., 1994; Velez-Pardo *et al*., 1998). 5-HT is released throughout the brain by a diffuse modulatory system (see Figure 1b) which originates in the raphe nuclei of the brain stem and projects to the hypothalamus and limbic structures, involved in emotion, through the medial forebrain bundle.

Central serotonin activity has a diurnal rhythm, with higher 5-HT release in the hippocampus during the active period (Kalén *et al*., 1989). The actions of 5-HT are generally inhibitory and are mediated by G-protein coupled receptors (GPCRs) prior to re-uptake into the axon terminal (see Section 1.1.4).

a.



b.

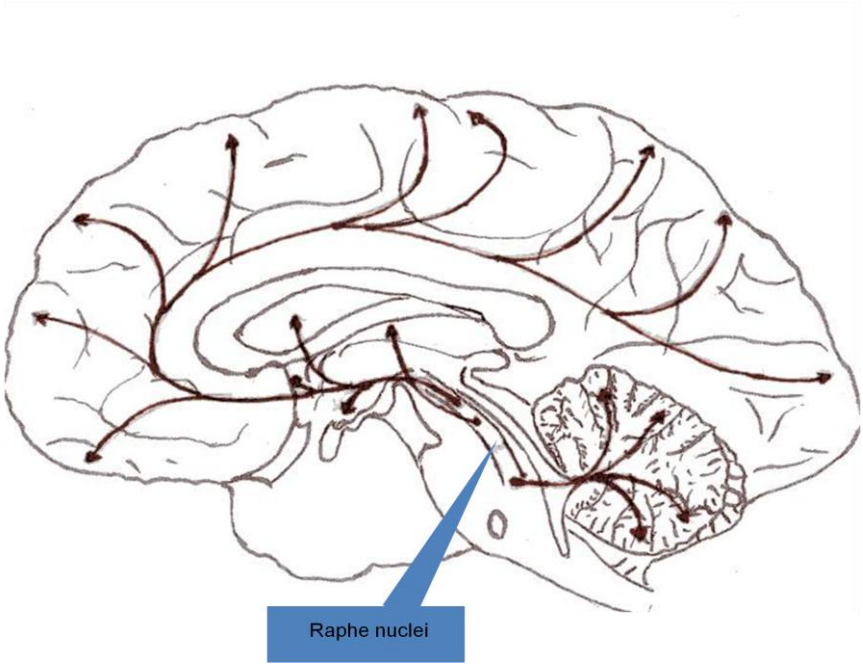


Figure 1.1: a) 5-HT production pathway (taken from internet)**; b) 5-HT projections throughout brain originating at the raphe nuclei**

1.1.2 5-HT Transporter

The serotonin transporter (SERT) reuptakes 5-HT in the synaptic cleft, regulating the concentration of 5-HT available and finally terminating the actions of 5-HT in the synapse. SERT gene has been localised to chromosome 17q11.1-q12 (Ramamoorthy *et al*., 1993), spans 31kb and consists of 14 exons (Lesch *et al*., 1994). The short variant polymorhism in the 5-HT transporter gene (SERTPR) is associated with anxiety and increased vulnerability to social stressors (Lesch *et al*., 1996; Grabe *et al*., 2005). In recurrent mood disorders the time course for improvement of core depressive and somatic anxiety symptoms by SSRIs is increased by the SERTPR polymorphism (Serretti *et al*., 2007). Another polymorphism of the SERT gene is a 44-bp insertion (L)/depletion (S) in the promoter region (SERTLPR). Controversial results on the association of the SERTLPR polymorphism and mood disorders have been found (Anguelova *et al*., 2003) and no association was detected recently between suicide and this polymorphism (Helbecque *et al*., 2006). Perturbation of the 5-HT system by disruption of the gene encoding the 5-HT transporter has region-specific compensatory alterations in 5-HT receptors. 5-HT_{1A} receptors were decreased in the dorsal raphe and increased in the hippocampus (Fabre *et al*., 2000; Li *et al*., 2000), 5-HT_{1B} receptors were decreased in the substantia nigra (Fabre *et al*., 2000) and 5-HT_{2A} receptors were decreased in the claustrum, cerebral cortex and lateral striatum (Rioux *et* al., 1999) of 5-HTT KO mice. The density of 5-HT_{2A} receptors (increased in hypothalamus and septum but reduced in striatum) and 5-HT_{2C} receptors (increased in amygdala and choroid plexus) are changed in a region-specific manner in 5-HTT KO mice (Li *et al*., 2003).

The high capacity plasma membrane monoamine transporter (PMAT) demonstrated capability to transport 5-HT and DA. mPMAT is expressed in neuronal cells but not in astrocytes and is co-expressed in many brain regions with the high affinity 5-HT transporter (SERT) and dopamine transporter (DAT), but it is also found in certain

sites that receive monoamine innervarion but lack significant expression of SERT or DAT (Dahlin *et al*., 2007).

1.1.3 Breakdown of 5-HT

MAO-A and MAO-B both breakdown 5-HT, but only MAO-B is present in serotonergic neurons. Although 5-HT is the preferred substrate for MAO-A, MAO-B is present in such a large excess thats its affinity for 5HT is substantial (Km = 1150µM, Fowler & Tipton., 1982). MAO-B can deaminate 5-HT in serotonergic neurons (Fagervall & Ross., 1986). MAO catabolises 5-HT (Berry *et al*., 1994) into 5-hydroxyindoleacetic acid (5-HIAA). The MAO-A has been mapped to Xp11.23 and is suggested to influence the mechanism of action of SSRIs through interaction with the 5-HT transporter (Sabol *et al*., 1998).

1.1.4 Action of 5-HT & Serotonin System Receptor Families

The classification of 5-HT receptors began in 1957 by Gaddum and Picarelli (Gaddum & Picarelli., 1957) when it was demonstrated that the effects of 5-HT, in guinea-pig ileum, could be partially blocked by morphine (M) and partially by dibenzyline (D). Two 5-HT receptor subclasses, 5-HT M and 5-HT D, were proposed to act by two different mechanisms with 5-HT M receptors suggested to be present in the nervous tissue while 5-HT D receptors were suggested to be in the muscles. In 1979, Peroutka and Snyder (Peroutka & Snyder; 1979) demonstrated two distinct populations of 5-HT receptors in rat brain using [^{3}H]5-HT, [^{3}H]spiroperidol and [^{3}H]LSD to label binding sites and these 5-HT receptor populations were designated 5-HT_1 ([^{3}H]5-HT binding) and 5-HT_2 (by [^{3}H]spiroperidol binding) with [^{3}H]LSD binding to both receptors to a similar extent. The M receptor, proposed by Gaddum, was found to be distinct from both the 5-HT_1 and 5-HT_2 receptors in function and distribution while the D receptor resembled the 5-HT_2 receptor pharmacologically. In 1986, Bradley *et al* (Bradley *et al*., 1986), proposed a framework for the classification of 5-HT receptors into 3 families based

primarily on functional criteria, named 5-HT_1-like, 5-HT_2 and 5-HT_3 (corresponding to the M receptor) receptors. Between 1987 and 1992 most known or suspected 5-HT receptors were cloned leading to the identification of a number of new receptors and subsequent revision of the classification of 5-HT receptor families. The new nomenclature system was proposed by the Serotonin Club Receptor Nomenclature Committee and was based on structural, operational and transduction information (Humphrey *et al.*, 1993). The current classification system is progressively adapted to incorporate new information from both recombinant and native receptors and to avoid species differences the nomenclature favours alignment with the human genome (Hoyer *et al.*, 1994; Hartig *et al.*, 1996; Hoyer & Martin 1997; Hoyer *et al.*, IUPHAR Receptor Database., 2006; doi:10.1786/543800763668). 5-HT receptors that are GPCRs belong to the type A family of GPCRs, contain the DRY motif in the third transmembrane spanning region and have significant sequence homology to adrenergic, dopaminergic and rhodopsin receptors (Hannon & Hoyer., 2008). At present there are seven families of 5-HT receptors that are classified based on pharmacology, transduction and structure with 14 different receptor subtypes (Barnes & Sharp., 1999). 5-HT receptors are membrane bound receptors (Peroutka & Howel., 1994) that interact with G-proteins to influence a variety of intracellular effects on systems with the exception of 5-HT_3, which is unique among monoamine receptors in functioning as a ligand-gated ion channel. Studies of these receptors have been complex and troublesome for many years due to the lack of selective pharmacological agents together with the nature of the receptors, such that they down-regulate upon ligand binding.

The 5-HT_1 receptor family comprises 5 receptor subtypes; 5-HT_{1A}, 5-$HT_{1B,}$ 5-$HT_{1D,}$ 5-ht_{1E} and 5-ht_{1F} which are encoded by intronless genes that are between 365 and 422 amino acids in length with an overall sequence homology of 40-63% (in humans). 5-HT_1 receptors are seven transmembrane GPCRs which are mostly linked to $G_{i/o}$ and are thought to be negatively linked to the adenylyl cyclase pathway leading to membrane depolarisation and inhibition of firing in cells. The 5-HT_{1D} receptor is located on chromosome 1p34.3 to p36.3; the product of the gene has 374

to 377 amino acids and is negatively coupled to cAMP production with a 61% sequence homology to the 5-HT_{1B} receptor (Hamblin *et al*., 1992). The 5-ht_{1F} receptor is less well characterised. This receptor is highly expressed in regions of the cortex, hippocampus, the caudate putamen and thalamic regions.

The 5-HT_{1A} receptor is situated on the human chromosome 5cen-q11.2-q13 (Hoyer *et* al., 1994) and is widely distributed in the CNS, in regions associated with control of mood such as the hippocampus, amygdala and septum, as well as being present in the PNS. 5-HT_{1A} receptor RNA is intronless and produces a polypeptide chain of 421 to 422 amino acids. No splice variants are known but two polymorphisms have been found which alter the extracellular amino terminal region of the receptor from Gly^{22} to Ser and from Ile^{28} to Val (Nakhai *et al*., 1995). This receptor preferentially couples to $G_{i/o}$ to inhibit adenylate cyclase activity (Hoyer & Schoeffter., 1991; Julius 1991) but has also been shown to be capable of activating the protein kinase C cascade (Fargin et al., 1991). Activation of the 5-HT_{1A} receptor causes neuronal hyperpolarisation mediated through G-protein-coupled K^+ channels. There is also evidence that in the presence of G_{i2} and adenylate cyclase type II, 5-HT_{1A} receptors stimulate cyclic AMP formation (Albert *et al*., 1999; Markstein *et al*., 1999). Agonist binding leads to G-protein mediated suppression of cyclic AMP production and enhancement of K^+-channel conductance (Lüscher *et al*., 1997).

In the raphe nuclei 5-HT_{1A} receptors are somatodendritic and act as autoreceptors causing a reduction in 5-HT synthesis, release into post synaptic sites and electrical activity (Blier *et al*., 1998; Richardson-Jones *et al*., 2010). It is proposed that desensitisation of the 5-HT_{1A} autoreceptors may underlie the ability of chronic, not acute, SSRI administration to raise synaptic cleft 5-HT (Invernizzi *et al*., 1994). In suicide victims that suffered from major depression, 5-HT_{1A} autoreceptors were increased suggesting decreased 5-HT activity (Stockmeier *et al*., 1998).

Anxiety and depression are associated with down-regulation of 5-HT_{1A} receptors (Gross *et al*., 2000) and disruption of the 5-HT_{1A} receptor gene increases anxiety-like behaviours (Heisler *et* al., 1998; Parks *et* al., 1998; Ramboz *et al*., 1998; Gingrich & Hen., 2001; Olivier *et al*., 2001) with transgenic re-expression of 5-HT_{1A} receptor in the hippocampus and amygdala during the early postnatal period being sufficient to rescue this behavioural phenotype (Gross *et al*., 2002). 5-HT_{1A} receptors play a role in some of the cognitive disturbances of schizophrenia, specifically attention, in response to antipsychotic drugs (Sumiyoshi *et al*., 2010). Agonists of this receptor induce spontaneous tail flicks in rodents (Bervoets & Millan., 1994, in rat). Motor coordination is not altered in 5-HT_{1A} receptor knock-out (KO) mice (Heisler *et al*., 1998).

The human 5-HT_{1B} receptor is located on chromosome 6q13, is G-protein coupled and is expressed in the CNS concentrated in the basal ganglia, striatum and frontal cortex (Hannon & Hoyer *et al*., 2008). 5-HT_{1B} receptors are expressed both pre and postsynaptically and act as autoreceptors in the 5-HT system (Hopwood & Stamford., 2001; Threlfell *et al*., 2010). The receptor has 386 to 390 amino acids and is negatively coupled to cAMP production (Schoeffter & Hoyer., 1989; Zgombick & Branchek., 1998). 5-HT_{1B} receptors are involved in aggressive behaviour with 5-HT_{1B} receptor KO mice reported to be highly aggressive (Ramboz *et* al., 1996) while 5-HT_{1B} receptor agonists potently suppress aggressive behaviour, food intake and sexual behaviour. An increase in anxiety and locomotion is found following activation of the 5-HT_{1B} receptors.

The 5-HT_2 receptor family exhibit 46 – 50% sequence homology and preferentially couple to $G_{q/11}$ increasing inositol phosphates and cytosolic [Ca^{2+}] and causing stimulation within the brain. Coupling may also occur through $G_{12/13}$ to mediate long term structural changes in cells. The 5-HT_{2A} receptor gene is located on human chromosome 13q13 to 13q21 and encodes for 471 amino acids in rat, mouse and humans. The 5-HT_{2A} receptor is highly expressed in the cortex, ventral striatum,

hippocampus, amygdala and basal ganglia and activation of this receptor stimulates the secretion of hormones such as ACTH and corticosterone (Van de Kar., 2001). The structures where 5-HT_{2A} receptors are expressed modulate anxiety-like behaviours (Millan *et al*., 2003). Global disruption of 5-HT_{2A} receptor signalling in mice reduces anxiety-like behaviour with selective restoration of 5-HT_{2A} receptor signalling in the cortex normalising behaviour suggesting a specific role for cortical 5-HT_{2A} receptor function (Weisstaub *et al*., 2006). Depressive-like or fear-conditioned behaviour is unaltered in 5-HT_{2A} receptor KO mice (Weisstaub *et al*., 2006). Other psychiatric disorders like anorexia nervosa and bulimia nervosa (Nacmias *et al*., 1999) and bipolar disorder (Bonnier *et al*., 2002) are associated with the 5-HT_{2A} receptor. 5-HT_{2A} receptors KO mice display reduced non rapid eye movement sleep expression (Popa *et al*., 2005).

The 5-HT_{2B} receptor is located on human chromosome 2q36.3 to 37.1 (Le Coniat *et al*., 1996) and encodes a 482 residue protein (Kursar *et al*., 1994). An alternative 5-HT_{2B} receptor has been reported (Bonhaus *et al*., 1995). Expression of this receptor is in the periphery (gut, heart, kidney and lung) and in the brain (particularly the cerebellum, dorsal hypothalamus and medial amygdala; Bonhaus *et al*., 1995). Activation of 5-HT_{2B} receptors causes anxiolysis and reduces grooming (Kennett *et al*., 1997; Duxon *et al*., 1997).

1.2 5-HT_{2C} receptor

5-HT_{2C} receptor could be a potentially important therapeutic target for the treatment of major health conditions including mood disorders, locomotor dysfunction and eating disorders (Gleason *et al*., 2001; Schuhler *et al*., 2005; Harada *et al*., 2008).

1.2.1 Background

The 5-HT_{2C} receptor has been mapped to human chromosome Xq 24 and is X-linked in both humans and mice. Expression of the 5-HT_{2C} receptor is of moderate density throughout the forebrain and hind brain, particularly in the choroid plexus. It is widely distributed in neocortical areas, hippocampus, nucleus accumbens, amygdala, dorsal striatum and substantia nigra (Lopez-Gimenez *et al*., 2007). Expression of the 5-HT_{2C} receptor is under the control of a monophasic diurnal rhythm with mRNA levels peaking shortly after the light phase begins (Holmes *et al*., 1997). Activation of 5-HT_{2C} receptors triggers signalling pathways which result in phosphatidylinositol (PI) turnover (Conn and Saunders-Bush., 1986; Conn *et al*., 1986). These receptors are able to couple to several G-protein α-subunits, including $G\alpha_q$, $G\alpha_{i/o}$, $G\alpha_{11}$, $G\alpha_{12}$ and $G\alpha_{13}$ (Bockaert et al., 2006; Cussac et al., 2002; McGrew et al., 2002; Price et al., 2001) allowing modulation of function of several intracellular signalling pathways. The best characterised signalling pathway modulated by the 5-HT_{2C} receptor is the $G\alpha_{q/11}$ phospholipase Cβ pathway (Conn *et* al., 1986; Qiu *et al*., 2007) which stimulates hydrolysis of phosphatidylinositol (4,5)-bis-phosphate (PIP2) leading to the accumulation of inositol triphosphate (IP3) that mobilizes intracellular Ca^{2+} and diaglycerol (DAG) which activates protein kinase C (see Figure 1.2).

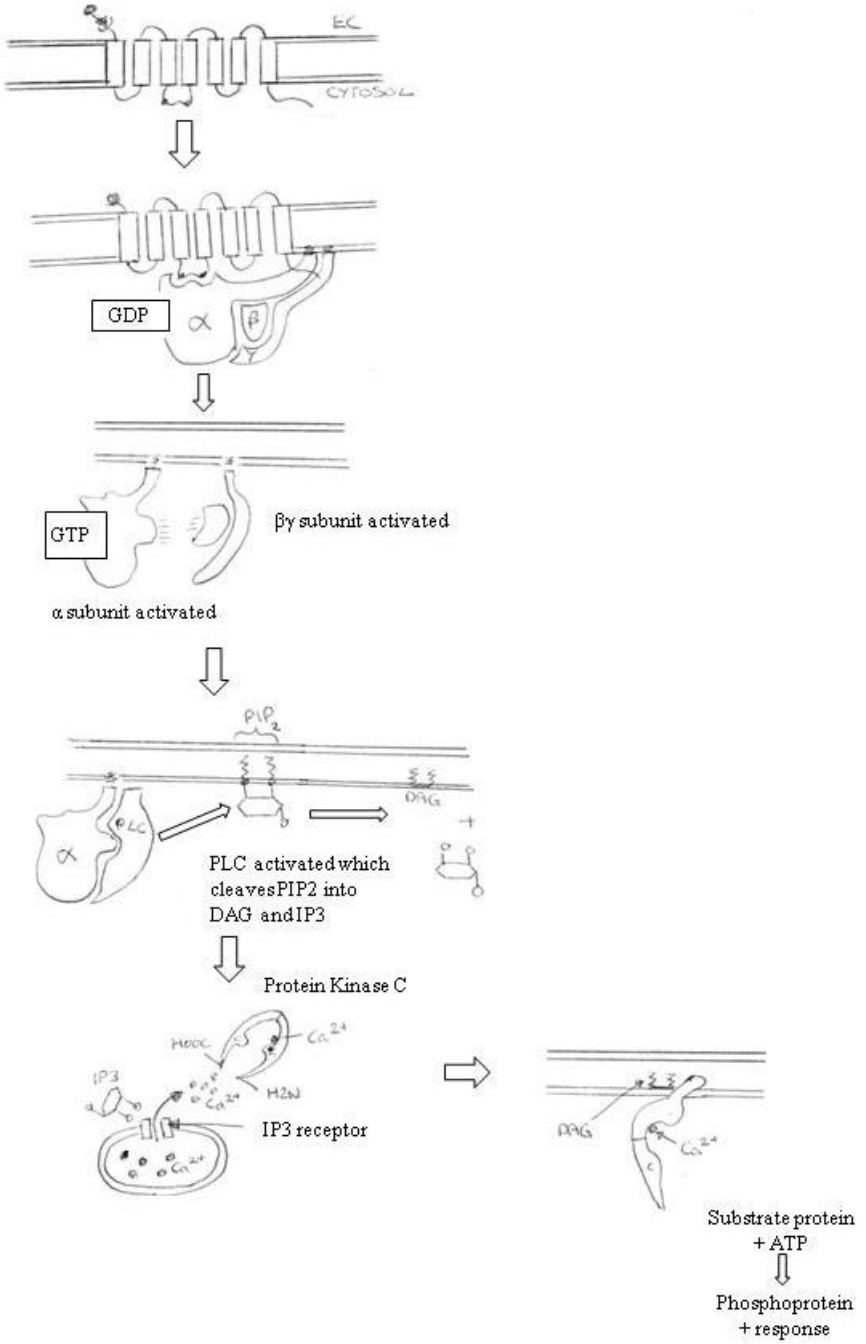


Figure 1.2: 5-HT_{2C} receptor activating the phospholipase Cβ pathway. The 5-HT_{2C} receptor becomes activated upon 5-HT binding and activates intracellular signalling pathways through coupling with G-proteins such as $G\alpha_q$. G-proteins consist of α, β and γ subunits. On activation of the 5-HT_{2C} receptor, $G\alpha_q$ binds to the second intracellular loop of the receptor. GDP is phosphorylated into GTP which activates the α subunit and exposes sites of the βγ subunit complex. The α subunit then activates phospholipase C which in turn cleaves PI 4,5 biphosphate (PIP_2) into DAG (which is in the membrane) and IP_3. IP_3 binds to the IP_3 receptor on the membrane of the endoplasmic reticulum which causes Ca^{2+} to exit the endoplasmic reticulum into the cytosol. The Ca^{2+} inds to protein kinase C causing activation and translocation to the membrane where it binds to DAG. The substrate protein in combination with ATP then forms phosphoprotein and generates a response.

1.2.2 Regulation of the 5-HT_{2C} receptor

Regulation of 5-HT_{2C} receptor is complex and involves many variables including 5-HT levels and exposure to stress (Prioleau *et al*., 2002; Maas *et al*., 2003; Gurevich *et al*., 2002a; Marion *et al*., 2004). At least two splice variants of the receptor have been found and intron editing occurs. 5-HT_{2C} receptors are also capable of post translational modification by A to I RNA editing of the exon, whereby adensosine residues can be represented as guanosine. This occurs in the second intracellular loop and yields at least eleven variants with differing affinities for binding 5-HT. Residues in the second intracellular loop connecting helices three and four in GPCRs have been demonstrated to play an important role in G-protein coupling. This editing occurs through ADAR 1 and 2 in the brain (Burns *et al.*, 1997; Wang *et al.*, 2000; Price *et al*., 2001; Flomen *et al*., 2004). Edited forms of the receptor require more ligand to be activated as well as coupling less efficiently to G-proteins (Burns *et al*., 1997, Fitzgerald *et* al., 1999, Niswender *et al*., 1999; Berg et al., 2008) suggesting unique signal transduction signatures *in vivo* dependent on the extent of editing. Editing at site C, A and B and/or D reduces 5-HT_{2C} receptor ability to activate phsopholipase C (Tohda *et al*., 2010) but editing levels of 5-HT_{2C} receptor were unaltered in mice lacking $G\alpha_q$ (Canal *et al*., 2009). Isoforms with a fully unedited genomic sequence have also been shown to have constitutive activity (Herrick-Davis *et al*., 1999, Niswender *et al*., 1999). The extent of editing also varies between genetically different mouse strains. The BALB/c strain, for example, encodes predominately the nonedited isoform which has the highest constitutive activity and agonist affinity compared to C57BL/6 which has ABCD or ABD edited isoforms constituting over 60% of its receptors (Englander *et al*., 2005). Editing levels may play a role in the pathophysiology of depression and the action of antidepressants with 5-HT_{2C} receptor editing being increased in depression (Dunlop et al., 2006) and depressed suicide victims were found to have significantly increased edited 5-HT_{2C} receptor isoforms (Niswender *et al*., 2001; Gurevich *et al*., 2002b; Schmauss., 2003; Dracheva *et al*., 2008a,b). Stress increases editing to isoforms which have reduced ligand sensitivity (Englander *et al*., 2005; Iwamoto *et al*., 2005) and early life stress

may permanently set this shift in editing levels accompanied by an increase in $G\alpha_q$ transcripts and protein in cerebral cortex (Bhansali *et* al., 2007). Depletion of 5-HT decreases the amount of editing and increases the pool of mRNA encoding receptor isoforms that have the highest constitutive activity and the highest affinity for 5-HT (Gurevich *et al*., 2002a).

Mice that express either the non-edited (5-HT_{2C}R-INI) or the fully-edited (5-HT_{2C}R-VGV) form of the 5-HT_{2C} receptor have recently been developed. No difference in signal density was found between 5-HT_{2C}R-INI and 5-HT_{2C}R-VGV mice suggesting unaltered levels of $G\alpha_q$ and $G\alpha_{11}$ protein (Canal *et al*., 2009). However, 5-HT_{2C}R-VGV mice have reduced ability to activate G-proteins which is masked by an increase in 5-HT_{2C} receptor binding sites in these mice (Olaghere da Silva *et al*., 2010). The 5-HT_{2C}R-VGV mice have phenotypic characteristics that are similar to that of Prader-Willi Syndrome, with decreased somatic growth, neonatal muscular dystrophy and reduced food consumption followed by hyperphagia after weaning (Morabito *et al.,* 2010). 5-HT_{2C}R-VGV mice have reduced fat mass and lipid accumulation in adipose tissue yet are hyperphagic compensated for by increased energy expenditure (Kawahara *et al*., 2008). 5-HT_{2C}R-INI mice have a depressive-like phenotype while complete editing of the 5-HT_{2C} receptor in 5-HT_{2C}R-VGV mice had antidepressant-like behaviour (Mombereau *et al*., 2010).

1.2.3 5-HT_{2C} receptor knock-out mice (5-HT_{2C} receptor KO mice)

Mice lacking the 5-HT_{2C} receptor were found to have a lowered seizure threshold and a more rapid progression of seizure activity, with 5-HT_{2C} receptor knock-out mice (5-HT_{2C} receptor KO mice) suffering from spontaneous seizures from the fifth postnatal week, resulting in death in hemizygous mutant male mice, with a 60% survival rate by 25 weeks (Tecott *et al*., 1995). These mice were created by insertion of a nonsense mutation into exon 5 of the cognate gene which placed a stop codon within the fifth putative transmembrane segment of the 5-HT_{2C} receptor, eliminating the carboxyterminal half of the 5-HT_{2C} receptor protein (Tecott *et al*., 1995). The mutation was confirmed using immunocytochemical analysis of brain sections and whole-cell voltage-clamp recordings in Xenopus oocytes injected with RNA from WT or mutant mice (Tecott *et al*., 1995). 5-HT_{2C} receptor KO mice had age dependent susceptibility to sound induced convulsions (AGS) with most KO mice displaying AGS by 75 days and complete penetrance by 120 days (Brennan *et al*., 1997). 5-HT_{2C} receptor KO mice implicated a role for 5-HT and 5-HT_{2C} receptor in the modulation of neuronal network excitability (Applegate & Tecott., 1998; Heisler *et al*., 1998). No alterations were found in 5-HT receptor subtypes (5-HT 1A, 1B/1D, 1F, 2A, 2B, 4 and 7) in 5-HT_{2C} receptor KO mice (Lopez-Gimenez *et al*., 2002).

1.2.4 5-HT_{2C} receptor and associated behaviours

The 5-HT_{2C} receptor is implicated in the regulation of food intake with non-selective agonists, such as mCPP, causing advancement in satiety and reducing food intake while specific 5-HT_{2C} receptor antagonists are able to block these anorectic effects

(Kennett & Curzon., 1988; Clifton *et al*., 2000; Vivkers *et al*., 2001; Hewitt *et al*., 2002; Schuhler *et al*., 2005). 5-HT_{2C} receptor KO mice were found to be hyperphagic and both plasma glucose levels and insulin were similar in KO compared to WTs when on a standard paired feeding analysis (Tecott *et al*., 1995). Due to larger white fat stores 5-HT_{2C} receptor KO mice develop obesity in later life but no alteration in the level of brown adipose tissue was found compared to WT. This hyperphagia and obesity is due to behavioural abnormalities as opposed to metabolic defects (Tecott *et al*., 1995). It has been shown recently as early as 10 days (before obesity develops) 5-HT_{2C} receptor KO pups are hyperphagic, hypometabolic and hypoactive but more competent at thermoregulation, choosing a warmer ambient temperature to defend core body temperature instead of expending metabolic energy (Akana *et al*., 2008). In a recent paper it was suggested that mis-timed and higher leptin levels during early life in 5-HT_{2C} receptor KO mice (increased leptin levels found at 16, 18 and 20 days old) could programme a change in both feeding and stress neurocircuits (Akana *et al*., 2008).

Table 1.1 Commonly used ligands affecting the 5-HT_{2C} receptor

Ligand	Behavioural effect	Receptors through which effect achieved
mCPP	- Reduced food intake - Reduced locomotion - Increased anxiety	5-HT receptors including 5-$HT_{1A/2B/2C}$ receptors
mCPP	- Increased locomotion in 5-HT_{2C} receptor KO mice	5-HT receptors excluding 5-HT_{2C} receptor
RO 60-0175	- No effect on anxiety	5-HT_2 receptor subtypes
RO 60-0175	- Decreased locomotion in 5-HT_{2C} receptor KO mice	5-HT_2 receptor subtypes excluding 5-HT_{2C} receptor
S32006	- Anxiolytic - Antidepressant properties	5-HT_{2C} receptor
Agomelatine	- Antidepressant - Improves sleep disturbances	5-HT_{2C} receptor and melatonin receptor
WAY163909	- Antidepressant	5-HT_{2C} receptor

The 5-HT_{2C} receptor is implicated in mood and has specific effects on anxiety, with agonists such as mCPP having an anxiogenic and hypolocomotor effect in novel environments while antagonists act as anxiolytics (Jenck *et al*., 1998; Kennett *et al*.,

1989; Lucki *et al*., 1989; Fone *et* al., 1996; Kennett *et al*., 1992; Harada *et al*., 2008). 5-HT_{2C} receptor KO mice had an anxiolytic phenotype (Heisler *et al*., 2007b) but more recent investigation showed no difference in anxiety-like or depressive-like behaviours (Hill *et al*., 2010). Alterations in 5-HT levels also play a role in mood with selective serotonin reuptake inhibitors (SSRIs) used as a treatment for major depression (Deshauer *et al*., 2008) and as effective in the treatment of panic disorder as tricyclic (TCA) antidepressants. (Bell and Nutt., 1998).

The 5-HT_{2C} receptor plays a role in the production of corticosterone through the regulation of CRF in hypothalamic regions (Heisler *et al*., 2007a), therefore influencing the HPA axis. 5-HT release and activation of 5-HT_2 receptors, particularly within the amygdaloid complex, have been associated with generating anxiogenic behaviours and emotional stress (Chaouloff *et al*., 2000; Kawahara *et al*., 1993; Stein *et al*., 2000) as well as facilitating conditioned fear in rodents (Graeff *et* al., 1997; Mora *et al*., 1997) and learned fear in humans (Graeff *et al*., 1996). The 5-HT_{2C} receptor has been shown to have an anxiolytic effect in conditioned anxiety (Davis *et al*., 1986) and activation within the basolateral amygdala (BLA) potentiates innate fear responses (Campbell and Merchant., 2003). Another point of interest is that the 5-HT_{2C} receptor is expressed on both the projection neurones and interneurons in the BLA (Stein *et al*., 2000) which could have opposing actions on GABA release (Rainnie *et al*., 1999; Stutzmann *et al*., 1999).

In addition to the 5-HT_{2C} receptor being involved in anxiety-like behaviour regulation, it is also associated with depressive-like behaviour however the literature on the effects of agonists and antagonists is conflicting. Antagonists, in addition to being anxiolytic, have antidepressive properties (Millan *et al*., 2006) such as the potent 5-HT_{2C} receptor antagonist, S32006 (Dekeyne *et al*., 2008). DBA/2N mice have lower brain 5-HT levels and are found to be insensitive to citalopram, a SSRI, but when a 5-HT_{2C} receptor antagonist is administered in combination with citalopram this restores its antidepressant effect (Calcagno et al, 2009). Other 5-

HT_{2C} receptor antagonists augment the actions of SSRIs (Cremer *et al*., 2004). Agomelatine, a 5-HT_{2C} receptor antagonist and melatonin receptor agonist (Auinot *et al*., 2003; Chagraoui *et al*., 2003; Millan *et al*., 2003), is effective in the treatment of major depressive disorder as well as SAD disorder (Millan *et al*., 2005; Kennedy & Emsley., 2006; Zupancic & Guilleminault., 2006; Pjerk et al 2006; Olié & Casper., 2007). The antidepressant effect of agomelatine is in part due to its actions on the melatonin receptors, and has been found to improve sleep disturbances (Thase 2000; Pandi-Perumal *et al*., 2009) which often accompany chronic mild stress and depression (Gorka *et al*., 1996). There is evidence that agonists of the 5-HT_{2C} receptor may also be efficacious antidepressants (Dunlop *et al*., 2006). As mentioned previously, there is a role for editing in the pathophysiology of depression and action of antidepressants as depressed suicide victims show increased editing of the 5-HT_{2C} receptor (Gurevich *et al*., 2002b).

Agonists that act on the 5-HT_{2C} receptor have been shown to cause hypolocomotion which can be blocked by antagonist administration (Gleason & Shannon., 1998; Stiedl *et al*., 2007). Administration of the mixed 5-HT_{2C} receptor agonist mCPP produces dose-dependent hypolocomotion (Fone *et al*., 1998, Gleason *et al*., 2001; Kennett & Curzon., 1988). 5-HT_{2C} receptor KO mice are hyperactive (Heisler & Tecott., 2000; Dalton *et al*., 2004) but on mCPP administration elicits hyperactivity (Heisler & Tecott., 2000; Dalton *et al*., 2004) which could be due to activation of 5-HT_{1B} and 5-HT_{2A} receptors.

1.2.5 5-HT_{2C} receptor ligands

In rodent studies, mCPP induces anxiety (Cornelio *et* al., 2007, Fone *et al*., 1996, Hackler *et* al., 2007, Kennett *et al*., 1992), reduces food intake (Hewitt *et al*., 2002, Kennett *et al*., 1988, Samanin *et al*., 1979) and inhibits locomotion (Lucki *et al*., 1989; Fone *et al*., 1998; Gleason *et al*., 2001; Stiedle *et al*., 2007). In 5-HT_{2C} receptor KO mice mCPP actually elicits hyperactivity (Heisler & Tecott., 2000; Dalton *et al*., 2004). The actions of mCPP are a combined result of its activation of

several serotonin receptor subtypes, including the 5-HT_{2B} receptor and 5-HT_{2A} receptor (Dalton *et al*., 2004, Lee *et al*., 2004, Nonogaki *et al*., 2003).

Recently, more selective 5-HT_{2C} receptor agonists, such as RO 60-0175, have also been shown to have a degree of action on other 5-HT_2 receptor subtypes (Damjanoska *et* al., 2003, Higgins *et* al., 2001, Martin *et al*., 1998, Porter *et al*., 1999). A sedative-like effect of RO 60-0175, with no effect on anxiety, has been reported (Kennett et al 2000). RO 60-0175 produced hypolocomotion in the 5-HT_{2C} receptor KO model (Fletcher *et al*., 2009).

1.3 5-HT system and 5-HT_{2C} receptor regulation of the DA system

It is believed that 5-HT exerts a tonic and phasic inhibitory control over both the mesolimbic and nigrostriatal DA systems (see Section 1.4.1). The substantia nigra (SN) receives dense serotonergic input and 5-HT_{2C} receptors are expressed in the cell bodies of GABA-containing neurones in both the SN pars reticulate and the VTA (Moga & Moore., 1997; Moyer & Kennaway., 1999; Di Matteo *et al*., 2001; Bubar & Cunningham., 2007). This positions the 5-HT_{2C} receptor to be a strong candidate through which 5-HT is able to regulate the DA system. 5-HT_{2C} receptor antagonists increase DA release in the nucleus accumbens and prefrontal cortex by increasing the firing rate and bursting activity of DA-containing neurons in the VTA (Di Matteo et al 2001; McMahon et al 2001; Blackburn *et al*., 2002; Di Matteo et al 2002; Higgins & Fletcher 2003; De Deurwaerdere et al 2004). Local administration of SB206553 (5-HT_{2C} receptor inverse agonist when administered to specific sites) into the striatum increased DA efflux in a dose-dependent manner (Alex *et al*., 2005). This suggests 5-HT_{2C} receptors in the dorsal striatum play an important role in the regulation of the nigrostriatal system. Under basal conditions 5-HT_{2C} receptor KO mice show no difference in DA levels in the nucleus accumbens or dorsal striatum

(Rocha *et al*., 2002). However, more recently, increased basal DA levels were shown in the nucleus accumbens of 5-HT_{2C} receptor KO mice (Abdallah *et al*., 2009). 5-HT_{2C} receptor agonists suppress both dopamine (DA) and noradrenaline (NA) pathways in the frontal cortex (Millan et al., 1998; Gobert et al., 2000).

Agomelatine enhances extracellular levels of DA and NA in the frontal cortex which reflects its antagonist properties at the 5-HT_{2C} receptor, reducing inhibition to ascending dopaminergic and noradrenergic pathways (Millan *et al*., 2000; 2003; De Deurwaerdére *et al*., 2004; Millan., 2005).

1.4 The Dopamine System

1.4.1 DA synthesis and projection pathways

In 1957, Carlsson showed that dopamine (DA) was a neurotransmitter in the brain (Carlsson., 1993) and not just a precursor for noradrenaline (NA). DA is a catecholamine neurotransmitter which plays many roles in behaviour such as reward, motivation and movement. It is synthesised by a two-step pathway with the first step being hydroxylation of L-tyrosine to L-DOPA by the enzyme tyrosine hydroxylase (TH). Tyrosine hydroxylase is the rate limiting enzyme in DA production and is present in DA synthesising cell groups which are situated within these three areas (i.e. VTA, SNc and retrorubral fields; O'Byrne et al 2000). L-DOPA is decarboxylated by dopa decarboxylase into DA (see Figure 1.3). DA receptors are also present on these synthesising cells which means that both pathways have autoreceptors which are thought to be the D2 receptor and possibly the D3 receptor to a lesser extent.

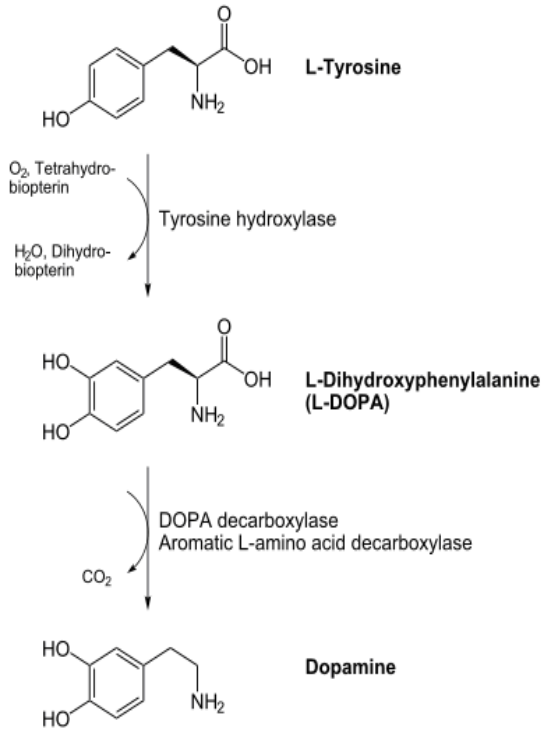


Figure 1.3: DA production pathway
DA is produced by a two step pathway, in which tyrosine hydroxylase is the rate limiting step (taken from internet)

There are two main DA projection pathways; the nigrostriatal system which projects from the substantia nigra pars compacta (SNc) to the STR and is involved in motor functions and the mesolimbic system which projects from both the retrorubral fields and the ventral tegmental area (VTA) to cortical and limbic structures, in particular the nucleus accumbens (see Figure 1.4a).

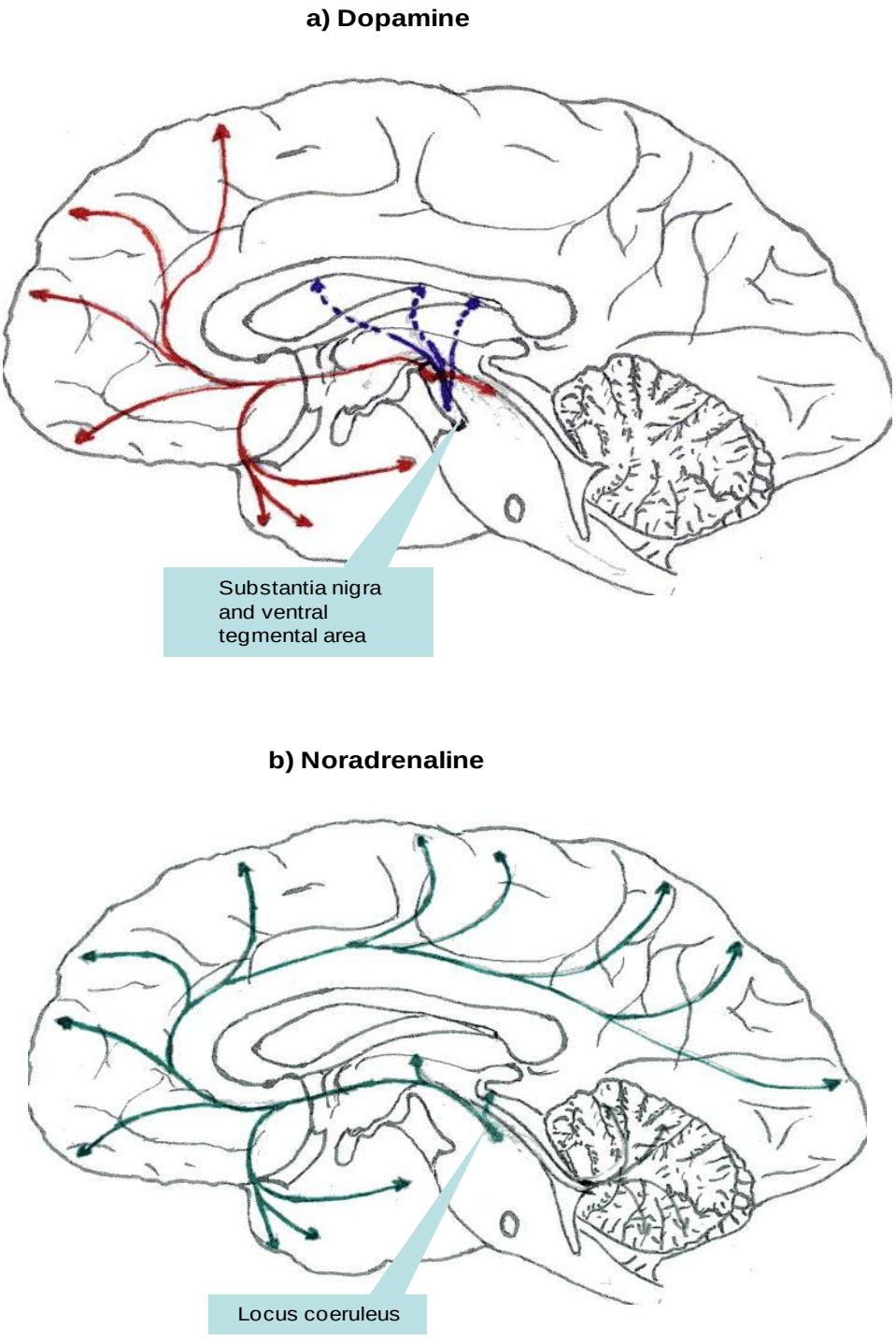


Figure 1.4: Catecholamine projection pathways
The two main DA projections pathways (a), the nigrostriatal (represented in blue) and the mesocorticolimbic (represented in red) project from the VTA and SN. The NA projection pathways (b) are represented in green and project from the Locus coeruleus.

1.4.2 Breakdown of DA

There are two degradation pathways for DA that both result in the production of homovanillic acid (HVA). The action of DA is either terminated by reuptake by the DA transporter (DAT1) and broken down by monamine oxidase (MAO) into 3,4-Dihydroxyphenyl-acetic acid (DOPAC) which is then converted to HVA by catechol-O-methyl-transferase (COMT; Cooper et al., 1991). Alternatively in regions where the DAT1 is sparse, the noradrenaline transporter (NAT) reuptakes DA, which is then broken down by COMT to 3-metoxytryptamine (3-MT). HVA is produced from the breakdown of 3-MT by MAO.

DA is also the precursor for noradrenaline (NA) which is synthesised from DA by dopamine β-hydroxylase which requires ascorbate as a cofactor (see Figure 1.5). The NA system originates in the locus coeruleus and the lateral tegmental field and projects to limbic regions, cerebral cortex and spinal cord (see Figure 1.4b). NA is involved in depression, influences the reward system and is involved in attention deficit/ hyperactivity disorder (Perona *et al*., 2008; Dell'Osso *et al*., 2010; Lim *et al*., 2010). In addition to its role in regulation of DA release, the 5-HT_{2C} receptor agonist, RO 60-0175, suppresses frontal cortex NA levels in rats while the 5-HT_{2C} receptor antagonist, SB-242084, increases NA levels. (Millan *et al*., 1998).

5-HT and noradrenaline reuptake inhibitors (SNRIs) are used in the treatment of some anxiety disorders and so increased concentration of NA at the synapse is predicted to have a beneficial effect on mood, including recovery of motivation (Dell'Osso *et al*., 2010).

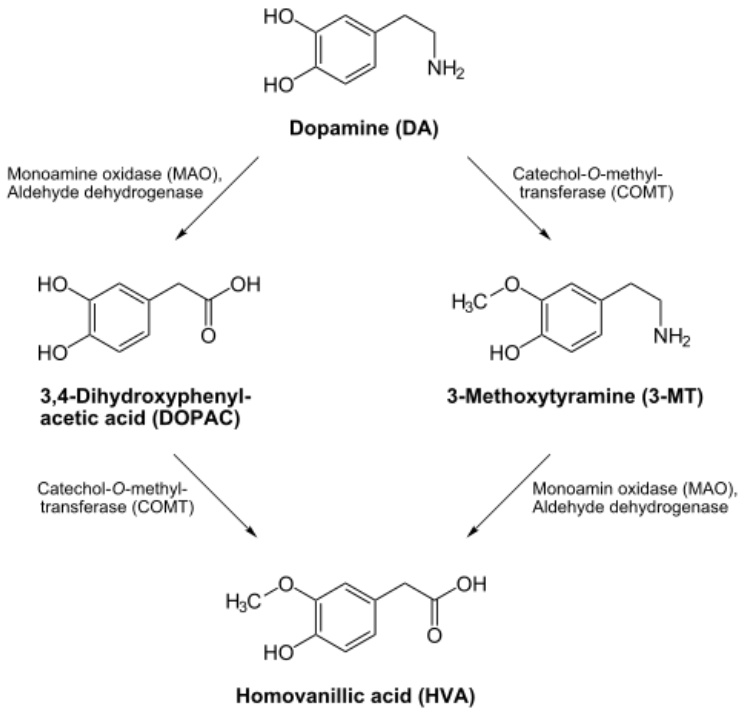


Figure 1.5: DA breakdown
DA can be broken down by one of two pathways, which both result in the production of HVA (taken from internet)

1.4.3 Action of DA & Dopamine system receptor families

At present there are two DA receptor families; the D1-like family includes the dopamine 1 and 5 receptors (D1; D5) which have similar pharmacological profiles and are coupled to adenylate cyclase through G-proteins. These receptors are intronless, have short third intracytosolic loops and short C-terminal tails. The D2-like family consists of the dopamine 2, 3 and 4 receptors (D2, D3, D4), each of which has unique pharmacological features. They are coupled to second messenger systems though inhibitory G-proteins. Due to the fact that they contain introns there

is the possibility of isoforms due to alternative splicing of their mRNAs and Drd2 and D3 receptor isoforms have been identified.

D1 and D2 receptors are homogeneously distributed in the dorsal striatum (STR), hippocampus and nucleus accumbens and are thought to play a role in motor and limbic functions (Yung and Bolam., 2000). D1 and D2 receptors are present on different populations of spiny projection neurones in the dorsal CPu. The direct pathway projects from the CPu to the substantia nigra and entopeduncular nucleus (the output nuclei of the basal ganglia) and the neurons that give rise to these projections have high levels of D1 receptor and the neuropeptides substance P and dynorphin. The indirect pathway projects from the CPu to the globus pallidus and the basal ganglia output nuclei indirectly and the neurons that give rise to this projection express high levels of D2 receptor and enkephalin (Drago et al 1994). D2 KO mice have reduced wakefulness (Qu *et al*., 2010) and reduced spontaneous movements in behavioural tests (Baik *et al*., 1995). The D3 receptor is expressed in the striatum and hippocampus but high expression is only found in the nucleus accumbens which suggests that it plays a role in limbic function (Suzuki et al., 1998), the nucleus accumbens shell responds sensitively to drugs of abuse suggesting a role for D3 receptors in addiction (Filip & papla., 2001).

1.4.4 Actions on behaviour with links to the 5-HT_{2C} receptor

Dopamine (DA) is of vital importance in many core behaviours including pleasure and movement. Dysfunction of this system can have devastating consequences as can be seen in Parkinson's disease where DA is depleted by a gradual degeneration of the substantia nigra pars compacta cells projecting to the striatum and results in a

loss of movement (Bossy-Wetzel *et al*., 2004). Depletion of DA signalling in the forebrain shown to cause a reduction in locomotion (Sedelis et al., 2001).

The DA system is implicated in mood disorders with DA system over-activity associated with schizophrenia and levels of HVA are increased in the plasma of schizophrenic patients (Arrúe *et al*., 2010). DA signalling is known to play a significant role in the reward mechanism that underlies some behavioural symptoms of depression when dysregulated (Naranjo *et al*., 2001). 5-HT and NA reuptake inhibitors (SNRIs) are used in the treatment of some anxiety disorders (Dell'Osso*et al*., 2010).

As well as being involved in some symptoms of depression, DA also plays a role in addiction, with DA release in the NA shell mediating motivation and reward (Volkow et al., 2004). 5-HT_{2C} receptor is implicated in the serotonergic suppression of DA-mediated behavioural responses to cocaine (Rocha *et al*., 2002). The reinforcing efficacy of cocaine is increased in the absence of 5-HT_{2C} receptor as self-administration is elevated in KO mice (Heisler & Tecott., 2000). A possible therapeutic use of the antidepressant, venlaxafine, in cocaine abuse has been suggested due to the ability of this antidepressant combined with a D3 receptor agonist to mimic the effects of cocaine in rats (Filip & Papla., 2001). There is also a role for the interaction of 5-HT_{2C} receptor and DA system in alcohol addiction (Pandey & Pandey., 1996; Thanos *et al*., 2005).

Both the 5-HT and dopamine systems are proposed to be involved in obsessive compulsive disorder (OCD). Reduced availability is found in the dopamine transporter in the striatum and 5-HT transporter in midbrain, brainstem and thalamic/hypothalamic in OCD patients (Hesse *et al*., 2005). It was found that selective serotonin reuptake inhibitors (SSRIs) alleviated symptoms of OCD in some cases and a recent study showed that treatment with SSRIs normalized the DA

function in the basal ganglia which correlated to a clinical improvement in compulsive symptoms (Kim et al 2007). 5-HT_{2C} receptor KO mice have been suggested to be a potential model for obsessive compulsive disorder. In a recent study the KO were found to have increased clay chewing and head dipping behaviour (Chou-Green et al., 2003). They also chewed plastic mesh screens in a 'neat' manner.

1.5 Circadian Biology

Circadian rhythms allow organisms to adapt to their environment, coordinate vital activities, such as feeding behaviour and the sleep-wake cycle, and coordinate physiological and biochemical mechanisms. These rhythms are controlled by an internal molecular clock which allows them to be maintained in the absence of external cues. In mammals this master clock is situated in the suprachiasmatic nucleus (SCN) of the hypothalamus (Ralph *et al.*, 1990) and synchronises the body's physiological activities. The master clock can be adjusted by internal stimuli, such as information regarding physiological and behavioural status, and by external stimuli such as light. Zeitgeber is the term given to an environmental signal that the clock can entrain to. The timing of peripheral pacemakers in other physiological systems is coordinated by the SCN (Guo *et al.*, 2006; Kohsaka & Bass., 2006).

1.5.1 Light entrainable oscillator

The LEO is located within the SCN and allows the endogenously generated circadian rhythm to maintain synchronisation with the exogenous light dark cycle. The retinohypothalamic tract (RHT), which utilises glutamate, enables photic stimuli to be relayed to the SCN. Light signals present during the early subjective night delay the clock whereas signals during the late subjective night produce a phase advance in the circadian rhythm.

The molecular mechanism underlying the master/circadian clock involves rhythmic transcription of clock genes. *Per1* and *per2* are rhythmically expressed in the SCN with levels peaking around midday. If light stimulation occurs during the night the mRNA levels of *per1* and *per2* rise after fifteen minutes, peak one to two hours later and return to baseline within three hours (*per2* slightly lagging behind *per1*). They are able to mediate the effect of light on the state of the clock and it is indicated that these genes are necessary for photic entainment (Albrecht *et al*., 1997; Miyake *et* al., 2000; Asai *et al*., 2001; Yan & Silver., 2002).

1.5.2 Serotonin system and Circadian Rhythms

Several neurotransmitters have the ability to phase advance the master clock via non-photic input pathways. Treatment with these neurotransmitters during the day decreases the midday rise of *per1* and/or *per2*. It is already established that 5-HT is one of the neurotransmitters with the ability to phase-advance the LEO via non-photic input pathways (Prosser *et al*., 1990; Kohler *et al*., 1999; Kalkowski & Wollnik., 1999), with various 5-HT receptors suggested to be involved (Kennaway *et al*., 1996; Gannon *et al*., 2009).

5-HT_{2C} receptor expression shows a monophasic diurnal rhythm (Holmes *et al*.,1997). The receptor is associated with sleep regulation, being implicated in both primary and secondary sleep disorders (Teegarden *et al*., 2008; Wafford & Ebert 2008) and 5-HT_{2C} receptor KO mice having disrupted sleep-wake periods and longer periods of wakefulness (Frank *et al*., 2002). Blockade of the 5-HT_{2C} receptor reduces REM sleep time and produces fewer nonREM – REM sleep transitions (Sharpley *et* al., 1994; Benington & Heller., 1995; Frank *et al*., 2002), while agomelatine, a 5-HT_{2C} receptor antagonist and melatonin agonist, improves sleep disturbances and is a potential treatment of seasonal affective disorder (SAD; Millan *et al*., 2005; Pjerk *et al*., 2006; Descamps *et al*., 2009; Pandi-Perumal *et al*., 2009). As previously

mentioned the 5-HT_{2C} receptor is involved in depression and the internal clock is profoundly disturbed by depression (Mendlewicz., 2009).

One potential mechanism through which the 5-HT_{2C} receptor might influence the circadian control of sleep is via regulation of the main circadian oscillator; the light entrainable oscillator (LEO) located in the suprachiasmatic nucleus (SCN) of the hypothalamus (Ralph *et al*., 1990). This region receives a dense serotonergic input from the raphe nucleus and 5-HT_{2C} receptor is present in the outer layer which puts it in a position to potentially influence circadian entrainment (Moyer & Kennaway; 1999; Varcoe & Kennaway., 2008). As previously mentioned two of the clock genes expressed in the SCN are *per*1 and *per*2 which are essential for rhythm responses to external stimuli such as light (Albrecht *et al*., 2001; Bae *et al*., 2001). The expression of both *per*1 and *per*2 is induced by a light pulse given in the dark phase (Albrecht *et al*., 1997; Miyake *et al*., 2000; Yan & Silver., 2002). Interestingly, systemic administration of a 5-HT_{2C} receptor agonist in the first half of the dark phase also induced *per*1 and *per*2 expression (Kennaway & Moyer., 1998; Kennaway *et* al., 2001; Varcoe *et al*., 2003). Activation of 5-HT_{2C} receptors in a denervated SCN in culture induced the expression of *per*1 indicating a post-synaptic mode of function (Varcoe & Kennaway., 2008).

1.5.3 Food Entrainable Oscillator

A second oscillator has been identified that entrains to food availability termed the food entrainable oscillator (FEO) of which the location (periphery or the CNS) is currently unknown. This was discovered as when the SCN is not functioning, either through a lesion or mutation such as the VPR2 KO mouse, food anticipatory running behaviour is seen when animals are fed at one time point each day. When food is presented outside the normal activity period the circadian rhythm regulated by the LEO and that of the FEO are dissociated. This results in two distinct behavioural components which can be monitored, one is the normal nocturnal activity regulated

by the master clock and entrained by the LEO and the other is food anticipatory running behaviour regulated by the FEO.

A body of literature documents the involvement of the 5-HT_{2C} receptor in regulation of food intake, with agonists being powerful appetite suppressants and some forms of obesity possibly associated with lowered 5-HT_{2C} receptor levels (Schuhler *et al*., 2005; Vickers *et al*., 2001). As food intake is influenced by the 5-HT_{2C} receptor and due to the 5-HT_{2C} receptor being expressed in a circadian manner (Holmes et al., 1997) it is possible that the 5-HT_{2C} receptor could influence the circadian eating pattern through signalling via the FEO.

Aims of Thesis

The main aims of this body of work were to:

1. Establish the levels of over- or under-expression of the 5-HT_{2C} receptor in the mouse lines and determine if a behavioural phenotype resulted.
2. Examine if there were compensatory alterations within the 5-HT system itself or in catecholamine levels in response to differing 5-HT_{2C} receptor levels
3. Determine if the 5-HT_{2C} receptor may play a role in the regulation of either the food entrainable oscillator or the light entrainable oscillator using the 5-HT_{2C} receptor deficient mouse line

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Unless otherwise stated, suppliers are located in the United Kingdom and all chemicals and reagents were purchased from **Sigma-Aldrich Company Ltd., Poole, Dorset.**

2.1.1 General Chemicals

Amersham Biosciences, Chalfont, Buckinghamshire:
NICK Sephadex G-50 DNA column®

Ambien:
RNase® Zap

BDH-Merck, Poole, Dorset:
Formaldehyde, Glacial Acetic Acid

Bio-Rad Laboratories:
Bio-Rad D_C Protein Assay Reagent A, Bio-Rad D_C Protein Assay Reagent B, Bovine Serum Albumin: Bio-Rad Protein Assay Standard II (1.48mg/ml)

Biowhittaker Molecular Applications, Wokingham, Surrey:
SeaKem® LE Agarose

Bright Instruments Co Ltd., Huntingdon, Cambridgeshire:
Cryo-m-bed Embedding Compound

Decon Laboratories Limited, Sussex:
Decon® 90

Fisher Scientific, Loughborough, Leicester:
Chromacol Vials (Clear screw vial, 9mm cap size), Duolite Mixed Resin, Hydrochloric Acid, Pre-cut silicone/PTFE caps 9mm, Sodium Sulfate ($NaSO_4$ Anhydrous)

Fluka Chemicals Ltd., Gillingham, Dorset:

Acetic Anhydride, Triathanolamine

Greiner Bio-one:

CELLSTAR® 96 well plate (flat bottom, with lid, sterile)

Invitrogen, Paisley, Renfrewshire:

ATP, CTP, Custom primers for genotyping, 1kb DNA ladder, DNase 1 amplification grade, dNTP mix, GTP, Low Melting Point Agarose, Random primers, RNase OUT, Superscript III reverse transcriptase, TRIzol®, Yeast tRNA

Macherey-Nagel, Düren, Germany:

NucleoSpin® Tissue

Merck Biosciences, Nottingham:

Dithiothreitol (DTT), LB broth

Millipore Corporation:

Nitrocellulose filter (0.22µm)

Promega Ltd., Southampton, Hampshire:

Buffer H, dATP, dCTP, dGTP, DNase 1 (RNase free), dTTP, HB101, Nucleus-free H_2O, Nucleotides, Restriction Enzymes (BamHI, EcoRI, HindIII, NcoI), RNA Polymerases (T7 and SP6), RNase Inhibitor (RNasin®), *Taq* Bead™ Hot Start Polymerase, Transcription Optomised Buffer (5x), Vectors (PGem-Teasy)

Qiagen Ltd., Crawley, West Sussex:

HotStar*Taq* DNA Polymerase, QIAprep® Spin Miniprep Kit, QIAquick Gel Extraction Kit, QIAquick PCR Purification Kit, RNeasy® Mini Kit

Roche Diagnostics Ltd., Lewes, East Sussex:

Complete mini protease inhibitor cocktail tablets, RNase A, Roche high pure PCR purification kit

Sigma-Aldrich Company Ltd., Poole, Dorset:

Costar SPIN-X® centrifuge tube filters (cellulose acetate membrane, pore size 22µm non-sterile), Kodak Biomax MR film

Viagen Biotech Ltd., Los Angeles, California, USA:

DirectPCR Lysis Reagent (Ear)

VWR International, Lutterworth, Leicester:

Acetone, Ethanol, NaH_2PO_4, Na_2HPO_4, Paraformaldehyde, SuperFrost Plus Slides

Whatman® International Ltd., Maidstone, Kent:

Whatman 3mm Chromatography Paper, Whatman Filter Paper

Zinsser Analytic, Maidenhead, Berkshire:

Aquasafe 300 Plus Scintillant

2.1.2 Radioisotopes

Amersham Biosciences, Chalfont, Buckinghamshire:

[N6-methyl-3H] Mesulergine (TRK 845), ^{35}S-UTP (30 TBq/mmol; 1.48 MBq/ µl)

2.1.3 Animals

Special Diet Services (SDS) Witham, Essex:

Rat and Mouse No.3 Breeding (RM3(E)) Chow (55.7% carbohydrate, 22.4% protein, 4.3% oil)

2.1.4 Injected Ligands

Sigma-Aldrich Company Ltd., Poole, Dorset:

mCPP (1-(m-chlorophenyl)-piperazine hydrochloride), SB 242084 dihydrochloride

Tocris Bioscience:

RO 60-0175 fumarate, SB 206553 hydrochloride

2.1.5 Equipment

Amersham Biosciences, Chalfont, Buckinghamshire:

GeneQuant RNA/DNA Calculator, Hypercassette™

Beckman Coulter Ltd., High Wycombe, Buckinghamshire:

Beckman Centrifuge Tubes, Beckman Optima™ TLX Ultracentrifuge, TLA 100.3 rotor

Bio-Tek Instruments Inc., Winooski, Vermont, USA:

EL 312e Bio-Kinetics Microplate Reader

Bio-Rad:

Bio-Rad Power Pac 200, Bio Rad Sub-Cell Model 96

Carl Zeiss, Welwyn Garden City, Hertfordshire:

Zeiss Stemi 100 Dissection Microscope

Dage Inc., Michigan City, Indiana, USA:

Dage MTI CCD72S Imaging Camera

Decon Ultrasenics Ltd, England:

Decon F5 Minor Sonicator

Denver Instruments:

pH meter

Eppendorf Ltd., Histon, Cambridge:

Centrifuge 5415R

Fuji Photo Film Company Ltd., Tokyo, Japan:

Phosphoimager FLA-2000, Phosphoimager screens

Heraeus Sepatech GmBH, Osterode, Germany:

Megafuge 3.0R

Hooke & Tucker Instruments, Croydon, Surrey:

Rotamixer

Hybaid Ltd., Basingstoke, Hampshire:

Hybaid Omni Slide Wash Module

Ika, Labortechnik, Staufen, Germany:

Ultra-Turrax T8 Auto-Homogeniser

Interfocus Imaging Ltd., Linton, Cambridgeshire:

Northern Light Illuminator

Jencons-PLS, East Grinstead, West Sussex:

Techne Dri-Block DB3, Techne Genius Thermal Cycler, Techne Hybridiser HB-1D, Techne Hybridisation Bottles

Konica Minolta, Milton Keynes, Buckinghamshire:

Konica SRX-101 X-ray Developer

Leica Microsystems Ltd., Milton Keynes, Buckinghamshire:

Leica Cryostat CM1900

Mettler-Toledo, Leicester:

Mettler HK60 Semi-Microbalance

Nikon, Japan:

OPTIPHOT-2 microscope

Packard Instruments, Berks, UK:

Packard tri-carb 2100TR

PerkinElmer, LAS (UK) Ltd., Beaconsfield, Buckinghamshire:

Wallac 1450 Microbeta Plus Scintillation Counter, Wallac Victor2 1420 MultiLabel Counter

Plastics Company, Edinburgh:

Elevated Plus Maze, Open Field

Schimadzu UK Ltd., Milton Keynes, Buckinghamshire:

Shimadzu UV-160A Spectrophotometer

Skatron Instruments, Lier, Norway:

Combi Cell Harvester

Spectronics Corporation, Westbury, New York, USA:

Spectrolinker XL-1500 UV Crosslinker

Starstedt, Numbrecht, Germany:

Microvette Tubes, EDTA coated

Technical & Scientific Equipment, Germany:

Food & Drink Cages

Techniplast UK Ltd., Kettering, Northamptonshire:

Mouse Wheel Running Cages

Ugo Basile, Comerio, Italy:

Passive Avoidance Apparatus, Rotorod

UVItec Ltd., Cambridge:

UVIpro Gel Documentation System

Waters Corpopration:

Waters 2475 Multi λ Fluorescence Detector Waters 2695 seperations module, Waters Sunfire 3.5µm C_{18} column

Weiss-Gallenkamp, Loughbourgh, Leicestershire:

Gallenkamp Fan Incubators

2.1.6 Software

Actimetrics Software, Wilmette, Illinois, USA:

ClockLab™, LimeLight™

InterFocus Imaging Ltd., Linton, Cambridgeshire:

MCID Analysis

Raytest Scientific Ltd., Sheffield:

Aida 2.0 Auto Image Data Analyser, Fujifilm Fluorescent Image Analyser FLA-200

Technical & Scientific Equipment, Germany:

Drink

The Mathworks Ltd., Cambridge:

MATLAB 6.5

UVItec Ltd., Cambridge:

UVIpro Acquisition Software

2.1.7 Buffers and Solutions

Box Buffer (100ml): 20ml 20x SSC buffer, 50ml deionised formamide and 30ml DEPC-H_2O.

Citrate Buffer: 0.1M sodium acetate (anhydrous), 0.1M sodium acetate tri-hydrate, 0.1M citric acid, 0.4mM octane sulphonic acid in HPLC grade water pH3.5. Stored at 4^{o}C and filtered through a 0.22µm nitrocellulose filter prior to use.

Deionised Formamimde: 150ml formamide mixed with 15g mixed bed ion-exchange resin (Duolite) for 2 to 3 hrs, filtered twice through Whatman filer paper and stored protected from light using sterile glassware.

DEPC-H_2O: dH_2O mixed with diethyl pyrocarbonate (DEPC; 1 drop/ 100ml), shaken , left for 30 mins, shaken again, and left for 1 hr prior to autoclaving.

1M Dithiothreitol (DTT): 0.15g was added to 1ml DEPC-H_2O and filter sterilised, stored at -20^{o}C.

1 kb DNA ladder loading mixture: 20µl ladder, 20µl loading dye and 160µl nucleus free water

250mM EDTA (pH8.0; 100ml): 80ml dH_2O was added to 9.3g $Na_2EDTA.2H_2O$. pH adjusted with NaOH and volume adjusted to 100ml. Autoclaved before use.

Ethanol in Ammonium Acetate:

50%: 11.55g Ammonium Acetate dissolved in 250ml ethanol, made up to 500ml with dH_2O.

70%: 11.55g Ammonium Acetate dissolved in 350ml ethanol, made up to 500ml with dH_2O.

90%: 11.55g Ammonium Acetate dissolved in 450ml ethanol, made up to 500ml with dH_2O.

2x Hybridisation Buffer: 1.2M NaCl, 20mM Tris-HCl (pH7.5), 2x Denhardt's solution, 2mM EDTA (pH8.0), 0.2mg salmon sperm DNA (11mg/ml), 0.2mg yeast tRNA (50mg/ml) and 2g dextran sulphate made up to 10ml in DEPC-H_2O, stored at -20^{o}C.

Loading buffer: 2g Ficoll® 400, 100mg SDS, 25mg bromophenol blue, made up to 10mls with 0.1M EDTA pH8.

4% Paraformaldehyde in 0.1M Phosphate Buffer (pH7.5): 20mM NaH_2PO_4, 80mM Na_2HPO_4 in 1L DEPC-H_2O, heated to 80^{o}C prior to addition of 40g

paraformaldehyde using sterile glassware. Stirred for 1 hr to dissolve and stored at $4^{oC.}$

1% PEI: 1ml polyethylenimine per 100mls water

10 x Phosphate Buffered Saline (PBS): 80g NaCl, 29g $Na_2HPO_4.2H_2O$, 2g KH_2PO_4, 2g KCl dissolved in 1L DEPC-H_2O. Autoclaved prior to use.

2 x Pre-Hybridisation Buffer: 1.2M NaCl, 20mM Tris-HCl (pH7.5), 2x Denhardt's solution, 2mM EDTA (pH8.0), 10mg salmon sperm DNA (11mg/ml) and 0.2mg yeast tRNA (50mg/ml) in DEPC-H_2O, stored at -20^oC.

Radiolabelled buffer: 50mM Tris-HCl, 10mM $MgCl_2$, 1mM EDTA and 50nM spiperone at pH7.4 with the addition of ~70,000 counts of ^{3}H-mesulergine.

RNase A Solution: 25mg RNase A (Roche) dissolved in 1mM Tris, 15mM NaCl made up to 2.5ml with dH_2O (10mg/ml). Heated to 100^oC for 15 mins then cooled to room temperature and stored at -20^oC. RNase A (10mg/ml) added to buffer (3µl/ml).

RNase Buffer: 0.5M NaCl, 10mM Tris, 1mM EDTA in dH_2O.

Room temperature buffer: 50mM Tris-HCl, 10mM $MgCl_2$, 1mM EDTA and 50nM spiperone at pH7.4

20 x SSC (Saline Sodium Citrate Buffer; pH7.0): UltraPure™ Gibco, Invitrogen

5M NaCl: 29.55g added to 100ml DEPC-H_2O.

0.32M sucrose-50mM Tris-HCl (pH7.4): 0.1% ascorbate containing protease inhibitors

10x TBE Buffer: 0.9M Tris base, 0.9M Boric acid, 20mM EDTA (pH8.0) in dH_2O. Autoclaved before use.

TE Buffer (pH8.0): 10mM Tris-HCl (pH8.0), 1mM EDTA (pH8.0) in DEPC-H_2O. Autoclaved before use.

0.1M Triethanolamine (pH8.0): 13.3ml Triethanolamine (Fluka) dissolved in 800ml DEPC-H_2O, adjust pH to 8.0 with HCl, adjust volume to 1L, using sterile glassware.

50mM Tris-HCl (pH7.4): 0.1% ascorbate containing protease inhibitors

5-HT Buffer: 50mM Tris-HCl, 10mM $MgCl_2$, 1mM EDTA, 50nM spiperone and 100µM 5-HT at pH7.4

2.2 Methods

2.2.1 Animals

Mice were maintained under conditions of controlled lighting (lights on 0700 -1900 h) and temperature (19-21^{o}C) and allowed *ad libitum* access to water and food (rat and mouse No 3, Special Diet Services, Witham, Essex, UK). All studies were performed to the highest standards of humane animal care under the aegis of the United Kingdom Animals Scientific Procedures Act, 1986 and approved by the ethics committee Edinburgh. For *in vitro* experiments, brains were taken from male mice (30-35g) after CO_2, and the tissue was immediately removed, frozen quickly on crushed dry ice and stored at -80^{o}C until use. For information on the generation of C2CR.10 and C2CR.33 mouse lines see Appendix 1 and for TetO-2CR mice see Appendix 2.

2.2.2 Genotyping

Ear notches were taken at time of weaning and tail tips at time of culling for genotyping. Both were stored at -20^{o}C until DNA extraction. Extraction was performed using separate reagent kits: NucleoSpin® Tissue (Macherey-Nagel) or DirectPCR Lysis Reagent (Viagen Biotech) with proteinase K. Genotyping was performed by polymerase chain reaction (PCR) using one protocol for C2CR.10 and C2CR.33 mice and another for TetO-2CR mice.

The following primers, corresponding to both the HA tag (5' forward primer) and complementary to the 5-HT_{2C} receptor cDNA (reverse primer) were used to genotype C2CR.10 and C2CR.33 mice. Forward primer: 5'-ATGTACCCATACGATGTTCCAGATTACGCT-3' and reverse primer: 5'-CAGAGGTGCATGGACGC-3'. This PCR was performed in a 50µl reaction mixture containing: 5µl buffer (from *Taq* Bead™ Hot Start kit), 3 µl $MgCl_2$ (from

Taq Bead™ Hot Start kit), 1µl dNTP mix (Invitrogen), 1 µl forward primer, 1 µl reverse primer, 38 µl filtered water, 1 µl DNA and 1 from *Taq* Bead™ Hot Start polymerase (Promega). Thermal cycling conditions compromised an intitial denaturation step at 96°C for 15 mins and 35 annealing and extension cycles of; 96°C for 45 secs, 62°C for 45 secs and 72°C for 1 min followed by a final extension step at 72°C for 10 mins in a Techne Genius thermal cycler (Jencons-PLS).

The following primers, corresponding to both the targeting cassette (5' forward primer) and complementary to the 5-HT_{2C} receptor coding region (reverse primer) were used to genotype TetO-2CR mice. Forward primer: 5' GCC CTT GGA ATT GAC GAG TAC G 3'and reverse primer: 5' GTG AGC CAA GAT TGT GCC ACT G 3'. This PCR was performed in a 50µl reaction mixture containing: 5µl 10xPCR buffer (Qiagen), 2µl dNTP mix (Invitrogen), 2 µl forward primer, 2 µl reverse primer (10µM), 0.5 µl HotStar*Taq* DNA polymerase (Qiagen), 36 µl filtered water and 0.5 µl DNA. Thermal cycling conditions compromised an intitial denaturation step at 96°C for 15 mins and 35 annealing and extension cycles of; 96°C for 45 secs, 62°C for 45 secs and 72°C for 1 min followed by a final extension step at 72°C for 10 mins in a Techne Genius thermal cycler (Jencons-PLS).

For all samples β-globin was used as a house keeping gene to detect the presence of DNA in all samples. The same programme was used as above but the primers were replaced with beta globin forward primer: CCAATCTGCTCACACAGGATAGAGAGGGCAGG and reverse primer: CCT TGA GGC TGT CCA AGT GAT TCA GGC CAT CG. The reaction mixture consisted of 5µl 5x buffer, 2 µl dNTPs, 2 µl forward primer, 2 µl reverse primer, 2.5 µl DNA, 0.5 µl HotStarTaq DNA polymerase and 36.5 µl filtered water.

5 µl 10 x loading buffer was added to 50 µl PCR products and 10 µl of this was loaded onto a 1% agarose (Biowhittaker Molecular Applications) gel containing 0.5x

TBE and ethidium bromide (1 µl/100ml gel). A 1kb DNA ladder (Invitrogen; 20 µl ladder, 20 µl loading buffer and 160 µl filtered water; 5 µl of ladder mix was loaded) was run to allow assessment of the size of the bands obtained. The gel was run at 100V until adequately separated and the results viewed on and photographed using the UVIpro gel documentation system (UVItec Ltd.). The presence of a 420 bp band indicates a C2CR.10 or C2CR.33 positive genotype.

2.3 Behavioural Testing of Mice

All behavioural tests were carried out between 0900 and 1300 hr, to coincide with the nadir of the corticosterone (CORT) circadian rhythm. Subjects were housed in a quiet room in groups of 2-3 mice with littermates. Mice were selected from within a cage in a random order for each test and the order of testing, for example mice that were tested last from a cage, did not alter the behavioural performance in the test.

Mouse cages were moved from the quiet holding room to the behaviour room 12 hr prior to the test for acclimatisation. Behavioural tests were carried out in a random order, except when they were scheduled for EPM testing. This was always carried out first as behaviour in this test was modified by previous testing. All apparatus was cleaned thoroughly with 90% ethanol between mice. The investigator was always blind to the genotype and/or drug treatment of the mice and all WT mice were littermate control mice used to limit the effect of the mixed genetic background during backcrossing onto C57BL/6.

2.3.1 Elevated Plus Maze Test

The elevated plus maze (EPM) is a common behavioural test that has been used extensively to investigate the psychological and neurochemical basis of anxiety and in the discovery of novel anxiolytic agents (Dawson & Tircklebank, 1995). The test has been validated in mice (Lister, 1987) and evokes an approach-avoidance response in rodents, based on their natural exploratory drive and their innate fear of open spaces. Thus, a highly anxious mouse will tend to avoid the open arms on an elevated plus-maze and spend the majority of time on the closed arms (Pellow and File, 1985, 1986).

The elevated plus-maze consisted of a plus-shaped platform made from black plastic and coated in white-sheeting, raised 1m above ground. One opposing pair of arms was enclosed by high walls (15.5 cm; closed arms) and the other opposing arms of the same size were exposed (open arms; 28cm). A central area of 6 cm^2 connected all four arms.

Each mouse was placed in the central area facing one of the open arms and allowed to explore the maze freely for 5 mins. During this time the behaviour of each mouse was monitored and recorded immediately using a computer tracking system, (Limelight version 2.2 software (Actimetrics, IL, USA)). The following spatiotemporal parameters were determined; number of open arm entries, time spent in the open arms and the distance travelled within the open arms. Ethological parameters such as stretch attend (stretching out from enclosed arms over the side of the open arm), rearing, grooming, immobility and faeces were also measured by the tester.

2.3.2 Open Field Test

The open field (OF) test measures the exploratory and anxiety-like behaviour in a large square novel environment and plays on the natural instinct of rodents to avoid open spaces. It was originally described in 1934 (Hall) and has since been comprehensively used in psychopharmacology (reviewed in Prut and Belzung, 2003).

The open field arena consists of a square box (50cm × 50cm, 25cm high, grey polymer, covered in white plastic sheeting) and was lit by two lamps (2 x 60W) which were angled at the centre. Limelight version 2.2 software (Actimetrics, IL, USA) was used to analyse the behaviour and movement of the mice. Mouse behaviour was continuously monitored by video camera and captured by Limelight™ Actimetrics software, the computer tracking programme, to allow for full spatiotemporal analysis. It superimposed three zones onto the floor of the arena to allow calculation of movement in separate areas of the field which as indicative of different types of behaviour. The zone adjacent to the wall represented a protected field, named 'outer zone', considered less anxiogenic than the inner zones which represented an exposed field, named 'inner zone'. C2CR.10, C2CR.33 or TetO-2CR and WT mice were tested during the first half of their light phase in their light/dark cycle, and each test was initiated by placing an individual mouse in the centre of the arena and allowing free movement for 5 minutes and on occasion this test was extended to 30 mins.

Parameters collected during each test comprised of the percentage distance covered in the inner zones, total distance covered in both zones, percentage time spent and percentage crossings in the inner zones. The ethological parameters recorded comprised of the following behaviours: outer zones rearing, inner zones vertical motions, grooming and the number of faeces laid during the test.

2.3.3 Sucrose Preference Test

TetO-2CR and WT mice were individually housed in a quiet behaviour room for one week prior the start of the test. Mice were given a choice of two-water bottles (labelled A and B) to drink from and throughout the test both water-bottles were weighed and their position switched daily to exclude preference for a particular bottle and any location bias. Once the mice had been trained to drink from both water-bottles (once preference for each water bottle was near 50% ±10%) the test was started and the fluid consumed was measured daily by weighing the bottles while the location of the bottles was continued to be switched daily. On days 1 and 2 both water-bottles were filled with water (w/w condition), days 3 and 4 both bottles were filled with a 1% solution of sucrose dissolved in drinking water (s/s condition) and on days 5-8 bottle A contained 1% sucrose solution above while B contained water (w/s condition, preference stage of test). The total fluid consumption was calculated as $[Vol_A + Vol_B]$ and averaged across the days of each bottle condition. The preference on each day for each mouse was calculated as $100(Vol_A/[Vol_A+Vol_B])$ and averaged across the days for each bottle condition (w/w, s/s or w/s). The values were body weight corrected for each mouse.

2.3.4 Tail Suspension Test

The tail suspension test is used to indicate depressive-like characteristics indicated by the time spent immobile during the test. Mice were individually suspended above the table top using an adhesive tape placed ~0.5 cm from the tip of the tail. Total duration of immobility was measured over a period of 6 minutes. Mice were considered to be immobile only when they hung down passively and were completely motionless. Mice were scored every 5 seconds as either S (struggling) or H (hanging). Hanging to struggling ratios, and percentage time spent hanging were also calculated.

2.3.5 Passive avoidance

The passive avoidance (PA) box (length - 39cm; width - 9.5cm; height – 16.5 cm; UGO Basile) features two chambers of equal sizes, both with gridded flooring. One chamber is made of white opaque polymer and is brightly lit, whilst the other is made of black polymer and is not illuminated. The two chambers are separated by a door which can be automatically opened and closed. On day one, mice were placed individually in the light chamber facing away from the doorway, which was open, and allowed to freely explore both chambers for 5 mins and the time spent in the light chamber recorded. On day two, mice were placed in the light chamber facing away from the closed door. After a 90 secs delay the door was opened and upon entering the dark chamber, the door closed and mice received an inescapable 0.3mA footshock for 3 secs and were left for a further 5 secs before being removed. This was repeated 6 hrs later, although no footshock was administered. The latency to enter the dark chamber during both trials was noted and the increased latency to enter following footshock represents a measure of fear-related memory.

2.3.6 Rotorod

The rotorod tests the general agility and balance of the mice and consists of a rotating rod which gradually increases in rotation velocity. Mice were individually placed onto the rod and given 10 secs to acclimatise. The rotorod speed was then continuously increased and at this point timing started. The trial ended when the mouse was unable to maintain balance on the rotorod (circled or fell off). The time measured from the velocity increase to the end of the trial was recorded.

2.3.7 Voluntary Wheel Running Behaviour

This protocol allows the level and pattern of activity to be measured for individual mice and therefore the effect of altered 5-HT_{2C} receptor levels on activity to be analysed. C2CR.10, C2CR.33 or TetO-2CR and respective WT control mice were individually housed in wheel cages (Techniplast; wheel diameter 23.5cm) and allowed to habituate to the environment. Wheel revolutions were monitored using Clocklab acquisition software (ActiMetrics, IL, USA) throughout the whole period. Data was averaged over the number of days of the test, excluding the habituation period (Clocklab analysis software (ActiMetrics, IL, USA)). 30 mins following *ip* administration of a drug wheel running activity was monitored for 1-2 hrs (coinciding with the first 1-2 hrs from the start of the dark phase) to determine drug-induced effects. The same time period from the previous day was used as basal data.

2.4 Circadian Experiments

2.4.1 Food and drink monitoring

TetO-2CR and WT mice were individually housed in food and drink monitoring cages and their food and drink intake measured every minute using Drink (Technical & Scientific Equipment). Average hourly intakes were calculated for the 24 hour cycle.

2.4.2 Light altered wheel running

TetO-2CR and WT mice were individually housed in wheel cages (wheel diameter 23.5cm) and wheel revolutions counted using Clocklab acquisition and analysis computer software (ActiMetrics, IL, USA) throughout the whole period. Average daily revolutions were calculated following an acclimatisation period of 7 to 14 days. Following a period of collection under normal light conditions the light cycle was altered from on 0700 off 1900 (14 days) to on 0300 off 1500 (7 to 14 days) then off constantly (12 to 24 days) before returning to on 0700 to 1900 (14 days).

2.4.3 Light pulses during wheel running

Following a period of acclimatisation under normal light conditions mice were subjected to a series of light pulses lasting 1 to 2 hours during the dark phase. Average hourly revolutions were calculated.

2.4.4 Food restriction in wheel cages

Following an acclimatisation period access to food was gradually restricted until it was presented at 11 am and removed at 5pm daily. Wheel rotations were counted for 14 days under food restriction before mice were put into constant darkness (9 to 14 days). Average hourly wheel rotations were calculated throughout.

2.5 Drug treatments

To elucidate the role of 5-HT_{2C} receptors in the behavioural tests, animals were injected with 5-HT receptor agonists (1-(m-chlorophenyl)-piperazine hydrochloride; mCPP, 0.3-3mg/kg; Sigma; (S)-2-(chloro-5-fluoro-indol- l-yl)-1-methylethylamine fumarate, RO 60-0175, 3-5mg/kg; Tocris Bioscience) 5-HT_{2C} receptor antagonist (6-chloro-5-methyl-1-[6-(2-methylpiridin-3-yloxy)pyridin-3-yl carbamoyl] indoline; SB242084; 3 mg/kg; Sigma), 5-HT_{2C} receptor inverse agonist (5-methyl-1-(3-pyridylcarbamoyl)-1,2,3,5-tetrahydropyrrolo[2,3-f] indole hydrochloride; SB206553; 1-5mg/kg; Tocris Bioscience), or vehicle (10% ethanol in saline for all drugs except mCPP which was dissolved in saline), via *i.p.* 30 min prior to testing or (for wheel running behaviour) 30 min prior to entering dark phase (i.e. 18.30h).

2.6 *In Situ* Hybridisation Histochemistry

In situ hybridisation allows for the visualisation of the structural location and quantification of specific mRNAs (indicating transcription of the corresponding gene) by hybridisation of a ^{35}S-labelled 'antisense' RNA probe to the mRNA of interest. ^{35}S-UTP labelled RNA 'sense' probes of similar length, nucleotide content and specific activity were included in order to assess the specificity of the hybridisation reaction.

To prevent the degradation of target mRNA by exogenous RNases only RNase free, sterile solutions and equipment were used for all *in situ* hybridisation experiments. All restriction enzymes and RNA polymerases were obtained from Promega UK Ltd.

2.6.1 Slide Preparation

To prevent section dehiscence, glass microscope slides were coated in 3-aminopropyltriethoxysilane prior to use. Slides were racked and washed in the following series of solutions; 0.2M HCl for 3 mins, DEPC-H_20 for 3 mins, 2% 3-aminopropytriethoxysaline in acetone (acetone dehydrated by filtering though $NaSO_4$) for 10 secs, acetone for 3 mins (twice) and finally DEPC-H_2O for 3 mins. Slides were air-dried for 30 mins before baking at 50^{o}C for 4 – 16 hr. Dried slides were wrapped in aluminium foil and stored at room temperature for up to 3 months.

2.6.2 Tissue Section Preparation

Whole brains were routinely frozen on crushed dry ice immediately after dissection from the animal in the first half of the light cycle and stored at -80^{o}C until required.

Frozen tissue sections were cut using a cryostat (Leica). Brains were later placed in the cryostat chamber at -18^{o}C and allowed to equilibrate for approximately 45 mins, slides were also put in to cool. Following equilibrium, brains were embedded in

Cryo-m-bed embedding compound (Bright Instruments) and positioned in the correct orientation for sectioning. 15µm thick coronal sections were collected and transferred to the prepared slides, and stored at -80^{o}C until required.

2.6.3 Preperation of probe templates

Templates were made by PCR from mouse brain RNA.

2.6.3.1 Identification of PCR primers for each gene

Regions of homology were determined for each gene by using the BLAST software (NCBI) with exon transcripts from Ensembl for dopamine transporter (DAT1), dopamine receptor 1 (Drd1), dopamine receptor 2 (Drd2), dopamine receptor (Drd3), 5-HT transporter and tryptophan hydroxylase 2 (TPH2). Possible primer sequences within mRNA sequences that were unique to that gene were identified using computer software (Primer 3) which allows the length of the requested primer sequences to be controlled in addition to the GC percentage content within the primer. The desired primers were 20 – 22 nucleotides long, had GC% of between 45 and 55%, ends of primer sequences were not complementary (to prevent primer dimmers forming) and primer pairs gave a product of between 300 and 600 bp. Each individual primer sequence and the product sequence were checked using BLAST to ensure the primers did not detect any other gene and that the product was specific for the gene of interest. Some primer sequences had to be selected by hand in order that they met the desired criteria. Primers were obtained from Eurogentec, Belgium.

2.6.3.2 RNA Extraction for PCR

Brains were removed from male mice before being frozen immediately in crushed dry ice. All samples were then stored at -80^{o}C until use. Samples were weighed and a maximum weight of 30mg transferred into a 2ml eppendorf and kept on dry ice. The element of the homogeniser had been soaked in a 10% solution of hydrogen peroxide in DEPC-H_2O for a minimum of 15 mins, then rinsed thoroughly in DEPC-

H_2O and wipe down with RNase free tissue. Total RNA was extracted using an RNeasy Mini Kit (Quiagen) and the standard protocol provided. RNA concentration was determined with a NanoDrop spectrophotometer (Thermo Fischer Scientific).

2.6.3.3 Preparation of cDNA templates by PCR

cDNA templates were prepared for various genes of interest, to be used subsequently for the synthesis of RNA probes for *in situ* hybridisation analysis. The cDNA was made using 1µg of RNA sample from the whole brain homogenate. The reaction mix contained 1 µl random primers, 1 µl 10mM dNTP mix and 11 µl diluted RNA (1 µg) and was incubated at 65^{o}C for 5 mins to linearise strands. Put on ice and then 4 µl 5x buffer, 1 µl 0.1M DTT, 1 µl RNase out and 1 µl superscript III were added and the reaction mix incubated at 25^{o}C for 5 mins, 50^{o}C for 60 mins (for enzymes to be active and stops coiling) and 70^{o}C for 15 mins.

A PCR was carried out on the cDNA for each set of primers (See Table for primer details). Each PCR had a reaction mixture consisting of 5 µl 5x buffer, 2 µl 10mM dNTPs, 2 µl forward primer, 2 µl reverse primer, 0.5 µl cDNA, 0.5 µl HotStartTaq polymerase and 38 µl NFW. The resulting cDNA PCR product was run through Roche high pure PCR product purification kit and the nanodrop was used to determine the concentration (ng/ µl). 200ng of each cDNA sample was run on an agarose gel, to check a clear band of the correct size was achieved. The PCR product was ligated into PGem-Teasy vector (Promega) using 1 µl T4 enzyme, 3 µl of purified PCR product and 1 µl vector (50ng/ µl) and 5 µl buffer by incubating for 4 hr at room temperature. 5 µl of each ligation incubated with HB101 on ice for 25 mins followed by a heat shock at 42^{o}C for 45 secs before being put back on ice. LB broth (300 µl) was added to each ligated culture and incubated at 37^{o}C for 1 hr in a shaker at 180rpm. Culture was pipetted onto ampicillin (100mg/ml) agar plates and incubated at 37^{o}C overnight. Ten colonies from each plate were selected to be added separately to 5mls of media with the addition of 5 µl ampicillin and incubated overnight at 37^{o}C in the shaker at 180rpm. Each culture was pelleted by a 5 min spin

at 2000rpm and then a QIAprep spin miniprep kit (Qiagen) used (45 µl elution buffer) to extract DNA from the bacterial preparation. The nanodrop was used to determine the concentration of each product (desired concentration was between 200ng/ µl and 400 ng/ µl) then 1 µl of each sample was incubated with 9 µl of master mix (64 µl 10x buffer H, 20 µl ECORI and made up to 576 µl with NFW) at 37^{o}C for 1 hr. Samples were loaded (with the addition of 2 µl loading buffer) and run on an agarose gel. All riboprobes cDNA with a distinct band were checked by sequence analysis (500 µl culture added to 500 µl glycerol to give 400ng per sample).

2.6.4 Linearising Riboprobe

A restriction digest was carried out to linearise riboprobes. The reaction mixture containing 6 µl template (10 µl for 5-HT_{1A} receptor), 4 µl buffer, 4 µl diluted BSA (1 µl BSA and 9 µl DEPC-H_2O), 22 µl DEPC-H_2O and 4 µl of the appropriate restriction enzyme (see Table 2.1) was incubated for 1 hr at 37^{o}C. To check the template was cut correctly 1 µl of each riboprobe was mixed with 2 µl loading buffer and 7 µl NFW and loaded onto a 1% agarose gel containing 0.5xTBE and ethidium bromide (1 µl/100ml gel). Each sample was then purified using a QIAquick PCR Purification Kit (Qiagen Kit) using 15 µl elution buffer and stored in 1.5 µl aliquots at -20^{o}C.

Table 2.1 Characteristics of Probe Generation for *In Situ* Hybridisation

Probe	Vector	Fragment size (bp)	Restriction Enzyme (AS)	RNA Polymerase (AS)	Temp (^{o}C)
1AR	pGEM Blue	910 bp	Hind III	T7	37
2AR	Blueskript SK	900 bp	Bam HI	T7	37
2CR	pGEM T easy	450 bp	Nco I	SP6	40
5-HTT	pGEM T easy	366 bp	Nco I	T7	37
TPH2	pGEM T easy	503 bp	Nco I	T7	37
TH	Pc DNA 3	400 bp	Eco RI	T7	37
Drd 1	pGEM T easy	592 bp	Nco I	T7	37
Drd 2	pGEM T easy	420 bp	Nco I	T7	37

0.5-1µg of linearised plasmid was transcribed by incubation at 37^{o}C (or 40^{o}C SP6 RNA polymerase) for 60-90mins in a reaction mixture containing 1mM ATP, CTP and GTP, 4 µl ^{35}**S-**UTP (1.48MBq/ µl; Amersham Ltd), 10mM DTT (Merck Biosciences), 0.5 µl RNase inhibitor (RNasin 40U/µl; Promega), and 1 µl appropriate polymerase (Promega) (Table 2.1) in a total volume of 10µl 5X transcription optimised buffer. Following incubation, the probe was cooled on ice before 1µl DNase l (RNase free) was added and reactions incubated at 37^{o}C for a further 15min to degrade the DNA template, after which probes were placed on ice for 1min and purified using NICK Sephadex column (Amersham Biosciences), to remove unincorporated radioactivity. The column was prepared by washing through with 3ml TE buffer (pH 8.0). 40µl of DEPC water was added to the probe mixture

before it was applied to the column. The column was then washed with 400µl TE buffer, and the initial elutant discarded, to wash off any unbound ^{35}S-UTP. The radiolabelled probe was eluted in an additional 400µl TE buffer.

For each probe, the total activity was estimated by counting 1µl of probe in 1ml PicoFluor 40 scintillant fluid in duplicate in a β-counter (Wallac 1450 MicroBeta Windows Workstation Version 4.01.014). The minimum activity required was $2x10^5$cpm/ l and all probes used were 10M/ml. Probes were stored at -20^{o}C until required, for a maximum of 7 days.

2.6.5 Fixation Protocol

Tissue sections (previously cut using a cryostat) were removed from -80^{o}C freezer and kept on dry ice until the start of the fixation procedure. Sections were fixed in ice cold 4% paraformaldehyde in 0.1M phosphate buffer (to maintain tissue morphology and inhibit endogenous ribonucleases; 2.76g NaH_2PO_4, 11.3g Na_2HPO_4 in 1 l DEPC-water, with 40g paraformaldehyde added at 80^{o}C) for 10min, rinsed twice in 1x phosphate buffered saline (PBS, 10x diluted from 10x PBS (80g NaCl, 29g $Na_2HPO_4.2H_2O$, 2g KH_2PO_4, 2g KCl in 1 l DEPC-water)) for 5min, acetylated in 0.1M triethanolamine with 0.25% acetic anhydride (to reduce non-specific binding of the probe to positively charged amino groups in tissues; 13.3ml triethanolamine in 1 l DEPC-water, pH 8.0, 0.75ml acetic anhydride added prior to transfer of slides) for 10min to prevent loss of signal and rinsed in lx PBS for 3min. Following dehydration through a series of ethanol solutions (70, 80 and 95% ethanol in DEPC-H_2O) slides were air dried for 30min.

2.6.6 Pre-hybridisation

Following fixation, slides were pre-hybridised with 200µl/slide buffer (2x pre-hybridisation buffer consisting of 1.2M NaCl, 20mM Tris-HCl pH7.5, 2x Denhardt's solution, 2mM EDTA, 10mg/ml salmon sperm DNA and 0.2mg/ml yeast tRNA in DEPC water diluted 1:1 with deionised formamide (Sigma)), at 50^{o}C for 2 hr. Dampening two layers of Whatman chromatography paper with box buffer (50% deionised formamide (deionised using 10% Duolite mixed resin), 20% 20 x SSC, 30% DEPC-water) humidified the slide boxes, hence preventing tissue sections from drying out.

2.6.7 Hybridisation

Sense and antisense ribo-probes were thawed and added to 50% 2x hybridisation buffer (1.2M NaCl, 20mM Tris-HCl pH7.5, 2x Denhardt's solution, 2mM EDTA, 0.2mg/ml salmon sperm DNA, 2g Dextran sulphate and 0.2mg/ml yeast tRNA in DEPC-H_2O plus riboprobe) diluted in 50% deionised formamide to give a final probe concentration of $10x10^6$ cpm/ml. Probes were denatured at 75^{o}C for 10min and placed on ice before addition of 10mM DTT. Pre-hybridisation buffer was drained from slides and 200µl of the appropriate probe was applied to sections. Slides were hybridised in sealed, humidified boxes (box buffer as above) at 50^{o}C for an optimum of 16 hours.

2.6.8 RNase Treatment to digest unbound ribo-probe

Following hybridisation, slides were washed three times in 2x SSC for 5min and carefully wiped dry around the sections with lens tissue. 200µl of RNase A (30mg/ml in RNase buffer, consisting of 0.5M NaCl, 0.01M Tris-HCl pH7.5, 1mM EDTA in DEPC-H_2O) was applied to each slide and left to incubate at 37^{o}C for 1 hr in humidified boxes (1 layer of Whatman No.3 chromatography paper dampened with RNase buffer as above) to remove unhybridised probe.

2.6.9 Washes and dehydration of sections

Following RNase treatment, slides were washed in 2x SSC at room temperature for 1 hr, once in 0.1x SSC at 60^{o}C for 1 hr min, and then once in 0.1x SSC at 60^{o}C cooling to ambient for 1 hr. After washes, slides were dehydrated through a series of ethanol solutions containing 0.3M ammonium acetate (2 mins in each of 50, 70 and 90% ethanol) and air-dried.

2.6.10 Visualisation of Hybridisation

Slides were exposed to autoradiographic film (Kodak Biomax-MR) and left for varying periods of time (1 to 14 days) depending on the specific activity of the probe used and the abundance of the gene transcript. Films were developed using Konica SRX-101 (JET X-ray, London).

2.6.11 Image Analysis

For image analysis, autoradiographic films were scanned on a high resolution flatbed scanner and any damaged or inadequate sections were excluded. The background was low with sense section backgrounds having a greyscale level not significantly different to zero. Digital image analysis was performed using MCID image analysis system (InterFocus Imaging) by optical densitometry. The appropriate section was selected per animal, 10 measurements were taken per area (five from each hemisphere), with the background subtracted. The specific signal was measured in several different regions specified in the individual chapters.

2.7 Radioligand binding assay for 5-HT_{2C} receptors

2.7.1 Development of technique

Firstly the spin conditions required to gain an optimal membrane preparation were tested and for this one large pooled WT mouse homogenate was used to remove individual animal variation while the technique was optimised. All spin fractions were tested to ensure the membrane sample contained all membranes. Varying membrane concentrations were then tested (0mg/ml up to 5mg/ml). The incubation temperature and time were also varied from 37°C for 15min, 30min or 45min, room temperature (~22°C) for 30min, 45min or 60min and at 4°C for overnight. A combination of methysergide and mianserin were used to block ^{3}H-mesulergine binding however using 5-HT was found to be more effective. Lastly the concentration of ^{3}H-mesulergine added to the incubate were altered varying from concentrations such as 5nM, 20nM and then by varying the counts of ^{3}H-mesulergine within the buffer varying from 70,000 to 140,000 counts.

2.7.2 Sample Preparation

Whole brains were removed from male mice and cut into forebrain, midbrain and hindbrain sections (for C2CR.10 mice the hindbrain was removed and the forebrain was left in one piece) frozen on dry-ice and stored at -80ºC until use.

Samples were homogenised in 0.32M sucrose-50mM Tris-HCl (pH 7.4), 0.1% ascorbate containing protease inhibitors (Mini Protease inhibitor cocktail tablets, Roche Applied Science, West Sussex, UK) then centrifuged at 300g for 5 min at 4ºC. The supernatant was removed and centrifuged at 50,000*g* for 15 min at 4°C. The resulting pellet was washed and resuspended in ice-cold 50mM Tris-HCl (pH 7.4), 0.1% ascorbate containing protease inhibitors for use in the assay.

The protein concentration of tissue supernatants were determined colorimetrically using a protein assay kit (Bio-Rad) and bovine serum albumin (BSA) standards (0.05-0.5 mg protein/ml). Samples were diluted in dH_2O to give concentrations in the standard range. Briefly, 25 µl Bio-Rad protein assay reagent A and 200 µl Bio-Rad protein assay reagent B were added to triplicate 10 µl aliquots of samples and standards in a 96-well plate. After 15 mins incubation at room temperature, the absorbance in each well was measured at 750nm (A_{750}) in a microplate reader. The concentration of protein in each sample was calculated from the standard curve (A_{750} vs. Mg/ml protein concentration). For the binding assay, samples were then diluted to 1.2mg/ml protein, which ends up as 0.6mg/ml per well. Samples were kept on ice at all times.

2.7.3 Membrane Binding

Total binding in membranes (0.6mg/ml protein) was determined in the presence of 10nM ^{3}H-mesulergine and 100nM spiperone (to block ^{3}H-mesulergine binding to 5-HT_{2A} receptors) while non-specific binding was determined with the addition of 100µM 5-HT. 96-well plates were first loaded with 25µl of either buffer (room temperature buffer containing 50mM Tris-HCl, 10mM $MgCl_2$, 1mM EDTA and a final concentration of 50nM spiperone, pH7.4) or 5-HT buffer (same as buffer above with the addition of final concentration of 100µM 5-HT) before 50µl of each sample (1.2mg/ml) was added in quartet, mixed gently and left to incubate at room temperature for 20mins. 25µl of radioloabelled buffer (same as buffer above with the addition of ~70,000 counts of ^{3}H-mesulergine) was added before an incubation at 37^{o}C for 30 min. Membrane incubation was terminated by rapid filtration using a Combi cell harvester (Skatron Instruments, Lier, Norway) onto a filter sheet that had been soaked in 1% PEI for 15 min. Filters were washed and left to dry for at least 1-3hrs. 25µl of the prepared radiolabelled buffer was put into vials, in triplicate, for counting to determine the number of counts of ^{3}H-mesulergine used in the incubations.

2.7.4 Counting Specific Binding

3mls of scintillant fluid (Aquasafe 300 Plus Scintillant) was added to each sample (and the vials containing 25µl radiolabelled buffer to determine counts of ^{3}H-mesulergine) and left to incubate for 6 hr at room temperature. Samples were counted on a liquid scintillation counter (Packard tri-carb 2100TR, Packard Instruments, Berks, UK) for 5 mins per samples. Samples were arranged to be counted in order with their radiolabelled and 5-HT buffer sample ordered for counting following on from one another to eliminate the incubation time in the scintillant fluid from having an effect on the result.

2.8 High Performance Liquid Chromatography (HPLC) of brain tissue

HPLC grade solutions were used throughout, and all solutions were filtered through a 0.22µm size filter using a vacuum filter.

2.8.1 Development of HPLC running conditions

Standard solutions of each compound being measured in the sample were required to develop the chromatography run conditions. 1mg/ml of each standard, 5-HT creatinine sulphate (5-HT, Sigma), 5 hydroxy-3-indole acetic acid (5HIAA, Sigma), dopamine 3-hydroxytryptamine (DA, Sigma), and the internal control methyl-5-hydroxytryptamine (CH3 5-HT, Sigma), were made dissolved in HPLC grade water, while noradrenaline (NA, Sigma) and homovanillic acid (HVA, Sigma) were dissolved in 0.1M HCl as they are unstable in H_2O. These stocks were stored at -20^{o}C. Each standard was run individually, and the retention time recorded, before standards were mixed together one by one and the percentage of methanol and temperature of column altered to ensure all standards had distinct retention times. A stock solution containing 0.3µg/µl of each standard in citrate buffer was prepared

and serially diluted 1:2 to obtain several solutions of known concentration (0.3, 0.15, 0.075, 0.0375, 0.01875, 0.009375, 0.004688, 0.002343, 0.001172, 0). The concentration of CH3 5-HT remained constant throughout standard curve dilutions and samples (0.3µg/µl). Ideally standards should also be diluted in PCA (instead of citrate buffer) to ensure all conditions are the same between the standards and samples.

2.8.2 Sample Preparation

Brains were removed from male mice and dissected into the hippocampus, cerebellum, cortex and the remainder, which contains thalamic regions, before being frozen immediately in crushed dry ice. All samples were then stored at -80^{o}C until use. Frozen tissues were weighed and homogenised in 200µl 0.1M perchloric acid (PCA), sonicated twice for 10secs and spun (10,000 g) for 5 mins at 4^{o}C. The supernatant was extracted and 0.3µg/µl of internal standard (methyl-5-hydroxytryptamine, CH3 5-HT, Sigma) was added before being filtered through a 22µm cellulose acetate centrifuge filter by application to a COSTARspinX column (Corning Life Science) and spun at 10,000 g for 1 min at 4^{o}C. A further 100µl 0.1M PCA was added to wash through the column and spun at 10,000 g for 1 min at 4^{o}C. Samples were kept on ice prior to chromatography and were run within 10 mins of preparation.

2.8.3 Running Samples

Samples were loaded into a Waters 2695 seperations module (Waters Corpopration) which autoinjected 10µl into a Waters Sunfire 3.5µm C_{18} column (Waters Corporation) with a mobile phase delivered at a constant flow rate of 0.4ml/min. Each sample took 35 mins to complete and the column was maintained at a consistent temperature of 25^{o}C. Natural fluorescence of the indole and catecholamines was detected by a Waters 2475 Multi λ Fluorescence Detector (Waters Corporation) at 284nm and 335 nm. The mobile phase consisted of 6%

methanol (BDH, HPLC grade) and 94% citrate buffer (pH3.5) filtered through a 0.22µm nitrocellulose filter (Millipore Corporation) and was kept in an isocratic mode throughout. A standard curve of all the standards was carried out to check retention times and throughout sample runs random standard curve points were selected to be checked.

2.8.4 Analysing data

Emission peak profiles for each sample were compared to those obtained from the standards in order to identify the catecholamine molecules of interest. The area of the emission peak was used to determine the concentration of each molecule from the standard curve. This was then corrected for the recovery rate, determined by comparing the mean internal control areas obtained from the samples to the mean internal control areas obtained from the standards. The concentration of each molecule in each sample was corrected by the tissue weight and volume of supernatant.

2.9 Statistical Analysis

Data are expressed as mean±SEM. Comparisons between groups were analysed by the most appropriate method including either an unpaired t-test, one and two-way analysis of variance (ANOVA) or a repeated measures (RM) one-way ANOVA, with Tukey's multiple comparison test or Bonferroni *post-hoc* testing; $p < 0.05$ was considered significant. For the HPLC data ANOVA were used to analyse the data as it was felt that T-tests were not valid in this case.

Appendix 1: Generation of transgenic mice overexpressing 5-HT_{2C} receptors in forebrain

This mouse line was generated by Gavin MacColl. The full length (unedited) rat 5-HT_{2C} receptor cDNA was amplified by PCR from a plasmid template (pMV7-sr1cm gift from David Julius, San Francisco, USA). An influenza hemagglutinin (HA) epitope tag was added at the N-terminus of the receptor by subcloning a double stranded oligonucleotide encoding the HA tag at a *Kpn*I site generated by PCR at the translation start of 5-HT_{2C} receptor cDNA. Receptor function is not altered by the N-terminal addition of this tag (Hurley *et al.*, 1999). The HA-5-HT_{2C} receptor cDNA was then subcloned into the *EcoRV* site of pNN265 (Mayford et al., 1996). Transient transfection of this plasmid into Cos7 cells conferred binding of the 5-HT_{2C} receptor ligand ^{3}H-mesulergine (data not shown). A *Not1* fragment including HA-5-HT_{2C} receptor (HA-2CR) cDNA was then inserted into the NotI site of pMM403, encoding the mouse calcium-calmodulin-dependent protein kinase (CamK) IIα promoter (Mayford *et al.*, 1996), to create the transgene construct, CamKIIα-HA-2CR (Figure 2.1). All constructs were verified by DNA sequencing. Transgenic mice were generated by pronuclear injection of the 11kb linearised CamKIIα-HA-2CR minigene construct into C57BL/6J x CBA embryos. Four founder mice (designated C2CR) were obtained, all of which transmitted the transgene. Two C2CR lines (C2CR.10 and C2CR.33) were selected for further characterisation, with similar results between lines, and mice were backcrossed to C57BL/6. In some experiments, genotypes were confirmed by southern blot analysis of *EcoRV* digested genomic DNA from C2CR mice, probed with a ^{32}P-labelled DNA fragment comprising the 2.7kb *BamH1* fragment of the transgene. Southern blotting was also used to confirm a single integration site of the transgene in C2CR.10 and C2CR.33 mice, consistent with the observed ~50% transmission rate for the transgene. Gestation length is normal in both C2CR.10 and C2CR.33 mice with an average litter size of 7 pups in C2CR.10 mice and 6 pups in C2CR.33 mice. All controls were WT littermate control mice to control for generation during the backcrossing process onto C57BL/6.

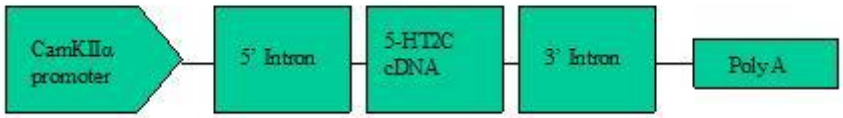


Figure 2.1: Transgenic construct
The over-expression of the 5-HT_{2C} receptor in the forebrain were generated by insertion of the above transgene containing 5-HT_{2C} receptor under the control of the CamK IIα promoter in a C57Bl/6/CBA cross. An haemagglutinin epitope tag was added at the N-terminus of the receptor which does not alter the function of the receptor. Due to the transgene containing 5-HT_{2C} receptor cDNA, no introns are present and therefore these receptors are not subject to A to I RNA editing. Male mice carrying the transgene were then bred with C57Bl/6 females to eventually have the transgene expressed on a C57Bl/6 genetic background.

Appendix 2: Generation of Targeted Mice Underexpressing 5-HT_{2C} receptors throughout brain

The targeting cassette used to generate this mouse line was named triTAUBi and was obtained from John Adleman, of the Vollum Institute, Portland, Oregon (Bond *et al*., 2000). This construct had a tetracycline activator (TetA) following the translation start, neomycin (NEO) resistance and URA3, which is used for selection in yeast. The construct was modified by Gavin MacColl, who replaced the URA3, of the original cassette, with an HPRT minigene obtained from David Melton (Moore *et al*., 1995) before homology arms were put on either side of the cassette for the 5-HT_{2C} receptor gene (5' length 1.8kb and 3' length 7kb). A 129-derived embryonic stem (ES) cell line was microinjected with the adapted targeting cassette by David Melton and following homologous recombination (see Figure 2.2), cells containing the correctly incorporated targeting construct were identified using Southern blot analysis by Karen French and Gavin MacColl. Following this selection the cells were microinjected into blastocysts and put into pseudo pregnant females and the chimera offspring were back crossed onto a C57BL/6 background creating C57BL/6 generation 1 of TetO-2CR mice. Additional DNA in the construct can decrease and even inhibit the action of the tetracycline (Tet). The HPRT minigene had floxP sites on either side which allowed it to be removed from the cassette by mating TetO-2CR offspring with a CRE-deleter mouse obtained from David Melton. This mouse line was actually found to under-express 5-HT_{2C} receptors and so TetO-2CR mice were used as an under-expressing model.

Due to the CRE-deleter mouse being of an undefined genetic background these offspring were backcrossed onto C57BL/6 to generate C57BL/6 generation 1 of

TetO-2CR mice. In all experimental procedures wild-type (WT) littermate mice were used to limit the effect of the mixed genetic background while backcrossing was taking place.

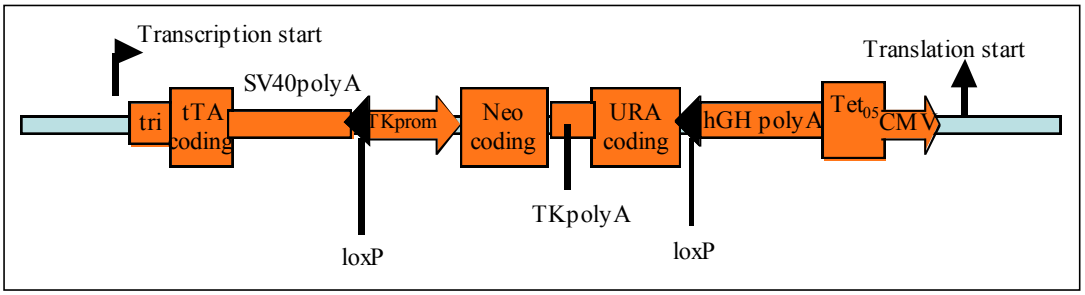


Figure 2.1: TetO-2CR mice targeting cassette inserted into 5-HT_{2C} receptor gene
The cassette encodes the binary tetracycline controlled trans-activator (tTA) protein controlled by a preceding adenovirus tripartite leader sequence that confers efficient translation initiation and followed by the polyadenylation/transcriptional termination sequence from SV40. The next portion of the cassette contains the bacterial neomycin resistance gene driven by the herpes simplex virus thymidine kinase promoter and the yeast URA3 gene. These selectable markers are flanked by loxP sites and followed by the polyadenylation/transcription termination sequence from the human growth hormone gene. The final part of the cassette contains 5 copies of the tet operator fused to the minimal cytomegalovirus (tet_{o5} CMV) promoter. The native (5-HT_{2C}) promoter drives expression of the tTA that induces transcription of the 5-HT2C gene from the tet_{o5} CMV promoter.

CHAPTER 3:
BASIC ANALYSIS OF TRANSGENIC MICE OVER-EXPRESSING 5-HT_{2C} RECEPTOR IN THE FOREBRAIN

Aims

This experimental chapter examines the 5-HT_{2C} receptor over-expressing mouse lines and has the following aims:

1. To fully validate the 5-HT_{2C} receptor expression pattern and levels in C2CR.10 and C2CR.33 mice
2. To establish any behavioural phenotype that may be present due to 5-HT_{2C} receptor over-expression
3. To examine functionality of the 5-HT_{2C} receptors in these models using ligand studies

It was hypothesised that 5-HT_{2C} receptor activity would be increased causing a reduction in locomotion and increased anxiety-like behaviour.

3.1 Introduction

The 5-HT_{2C} receptor could be a potentially important therapeutic target for the treatment of psychiatric diseases including mood disorders, locomotor dysfunction and eating disorders (Chapter 1; Gleason *et al*., 2001; Schuhler *et al*., 2005; Harada *et al*., 2008). It has been difficult to elucidate the precise function and pathophysiological role of the 5-HT_{2C} receptor for multiple reasons, in particular a lack of availability of selective pharmacological agents due to large regions of homology between 5-HT receptor subtypes. However, studies using 1-(m-chlorophenyl)-piperazine (mCPP), a non-selective 5-HT receptor agonist with preferential affinity for the 5-HT_{2C} receptor, show mCPP induces anxiety (Kennett *et al*., 1992; Fone *et al*., 1996; Cornelio *et* al., 2007; Hackler *et* al., 2007), reduces food intake (Samanin *et al*., 1979; Kennett *et al*., 1988; Hewitt *et al*., 2002) and inhibits locomotion (Lucki *et al*., 1989, Stiedle *et al*., 2007). Although these studies infer a role for the 5-HT_{2C} receptor in these functions, the actions of mCPP are a combined result of its activation of several serotonin receptor subtypes, including the 5-HT_{2B} receptor and 5-HT_{2A} receptor (Nonogaki *et al*., 2003; Dalton *et al*., 2004; Lee *et al*., 2004). Recently, more selective 5-HT_{2C} receptor agonists, such as RO 60-0175, have also been shown to have a degree of action on other 5-HT_2 receptor subtypes (Damjanoska *et* al., 2003, Higgins *et* al., 2001, Martin *et al*., 1998, Porter *et al*., 1999). Hence a definitive assessment of 5-HT_{2C} receptor function is not possible using pharmacological methods alone.

The 5-HT_{2C} receptor is under complex regulation of both receptor density and activity (Chapter 1). The 5-HT_{2C} receptor is also subject to A to I RNA editing of the pre-RNA transcript which alters its sensitivity to ligands (Niswender *et al*., 1999; Englander *et al*., 2005). *In vitro* edited forms of the receptor require more ligand to activate intracellular signalling pathways due to a coupling deficiency to G-proteins (Burns *et al*., 1997, Fitzgerald *et* al., 1999, Niswender *et al*., 1999) compared to the unedited form. The non-edited (genomic sequence) isoform exhibits constitutive activity (Herrick-Davis *et al*., 1999, Niswender *et al*., 1999) but the fully-edited isoform (VGV) does not. The extent of editing of the 5-HT_{2C} receptor varies

between genetically different mouse strains. The BALB/c strain, for example, expresses predominately the nonedited isoform which has the highest constitutive activity and agonist affinity compared to C57BL/6 in which the ABCD or ABD edited isoforms constitute over 60% of the receptors (Englander *et al*., 2005). Following early life stress, such as infant maternal separation, editing of the 5-HT_{2C} receptor is increased (Bhansali *et al*., 2007), while depletion of 5-HT decreases the amount of editing and increases the pool of mRNA encoding receptor isoforms that have the highest constitutive activity and the highest affinity for 5-HT (Gurevich *et al*., 2002a). A possible reason for 5-HT_{2C} receptor editing being altered following sustained alterations of serotonergic neurotransmission could be to maintain normal responses. Editing is altered in a site specific manner in human post-mortem brains from depressed suicide victims, indicating mood disorders can affect the pattern of 5-HT_{2C} receptor editing (Gurevich *et al*., 2002b).

The most appropriate way to examine the action of the 5-HT_{2C} receptor could be the development of a transgenic mouse model as even if truly specific ligands were to become available, you could not account for the other factors (such as A to I RNA editing) which could influence receptor function. In this study a transgenic approach has been taken to elucidate the effect of over-expressing the 5-HT_{2C} receptor in the forebrain by using mouse lines where the 5-HT_{2C} receptor is under the transcriptional regulation of the CamKIIα promoter to generate CamKIIα-5-HT_{2C} mice termed C2CR.10 and C2CR.33 mice (created by Gavin McColl). To minimise the effect of editing, the transgene was created from 5-HT_{2C} receptor cDNA which lacks introns and so is not subject to RNA editing. This created a model where (a) levels of 5-HT_{2C} receptor were increased and (b) the balance of edited isoforms should be considerably modified in favour of the non edited isoform compared to WT mice. The aim of this chapter is to determine whether increased 5-HT_{2C} receptor receptor expression results in behavioural dysfunction. A full validation of the receptor expression pattern and levels in each transgenic line will be carried out and compared to the behavioural outcome particularly locomotion and anxious-like behaviours to obtain unequivocal evidence for the 5-HT_{2C} receptor in these behaviours.

3.2 Initial characterisation of transgenic mouse lines

3.2.1 Transgene expression (indicated by 5-HT_{2C} receptor mRNA) was widespread in C2CR.33 mice but more restricted in C2CR.10 mice

The regions of the brain where the transgene had generated increased 5-HT_{2C} receptor mRNA expression was determined by *in situ* hybridisation. This technique allows semi quantification of 5-HT_{2C} receptor mRNA levels in brain sections and therefore clearly shows the expression pattern and level within these sections compared to WT mice (see Figure 3.0). Compared to WT mice, C2CR.10 mice (backcrossed to C57BL/6 for 2 generations) had significantly increased 5-HT_{2C} receptor mRNA levels in various regions of the forebrain from frontal regions moving to more posterior regions (Figure 3.1). Firstly in regions of the frontal forebrain such as the caudate putamen (CPu) and nucleus accumbens core (AcbC) and shell (AcbSh), C2CR.10 mice were found to have significantly increased 5-HT_{2C} receptor mRNA levels in the AcbC (Figure 3.1a; interaction of genotype and region: $F_{4,34}$=9.46; p<0.0001; effect of genotype: $F_{1,34}$=58.18; p<0.0001). In more posterior forebrain regions such as the dorsal hippocampus (CA1, CA3, DG), basolateral amygdaloid nucleus (BLA), cortex and choroid plexus (CP) there was a significant effect of the genotype of the mouse and its interaction with the regions tested (Figure 3.1b $F_{5,53}$=7.43; p<0.0001), with the transgene causing over-expression of 5-HT_{2C} receptor mRNA in the dorsal hippocampus (CA1, CA2/3 and DG) but not in the amygdala, CP or cortex. Finally, in sections containing ventral limbic regions, such as the cortex, ventral hippocampus (CA1, CA2, CA3, DG) and substantia nigra (SN) a significant interaction of the genotype and region tested was found (Figure 3.1c; $F_{5,54}$=4.08; p=0.003) with significant elevations in 5-HT_{2C} receptor mRNA in C2CR.10 mice compared to WT mice in both the CA3 field of the ventral hippocampus and the substantia nigra (SN).

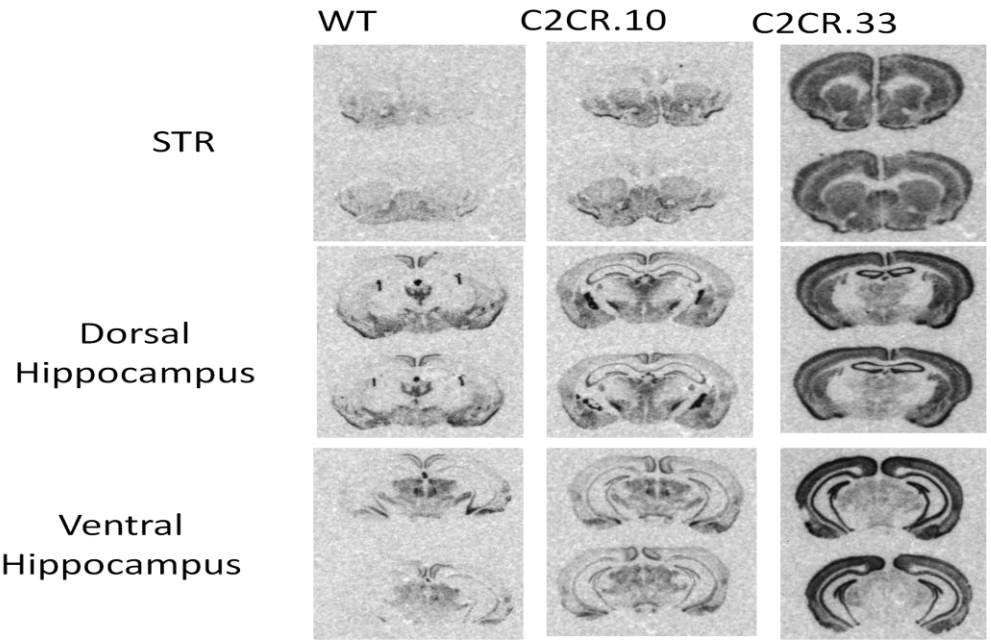


Figure 3.0: Example autorad photographs for 5-HT_{2C} receptor mRNA levels in C2CR.10 and C2CR.33 mice.
The differing levels of 5-HT_{2C} receptor mRNA can be visualised using autoradiographs.

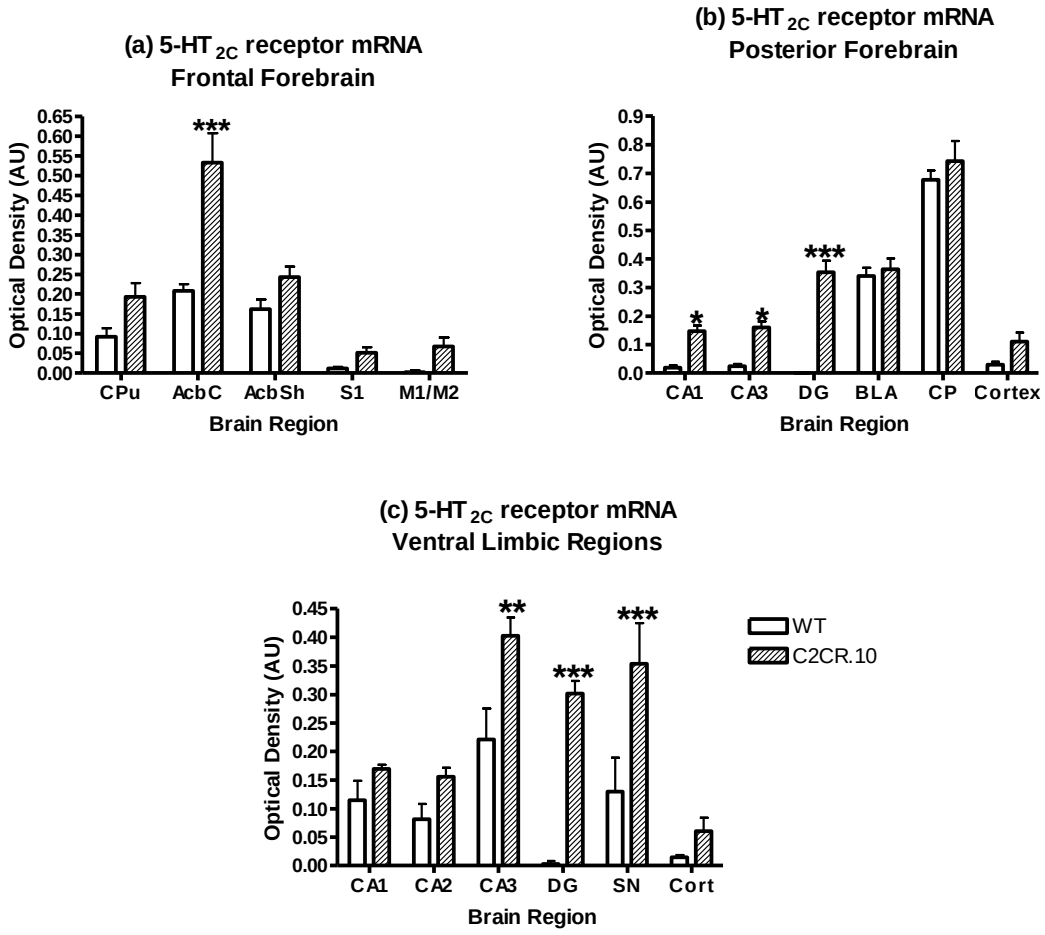


Figure 3.1: 5-HT$_{2C}$ receptor mRNA expression frontal to posterior in adult male C2CR.10 mice

5-HT$_{2C}$ receptor mRNA expression is increased in C2CR.10 mice compared to WT littermate control mice in (a) frontal forebrain, (b) posterior forebrain and (c) ventral limbic regions. Expression levels were measured by optical densitometry of *in situ* hybridisation autoradiographs. Data are expressed as mean±SEM in males backcrossed to C57BL/6 for 2 generations and analysed by unpaired, 2-way ANOVA with Bonferroni posttests; n= 5 per group. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

CPu = caudate putamen; AcbN = nucleus accumbens core; AcbSh = nucleus accumbens shell; S1 = primary somatosensory cortex; M1/M2 = primary/secondary motor cortex; CA1, CA2 or CA3 = CA1, CA2 or CA3 field of the hippocampus; DG = dentate gyrus; BLA = basolateral amygdaloid nucleus (anterior); CP = choroid plexus; SN = substantia nigra.

C2CR.33 mice backcrossed to C57BL/6 for 2 generations also had significantly increased 5-HT_{2C} receptor mRNA levels compared to WT mice across the forebrain (Figure 3.2). This increase was considerably more dramatic than that observed in C2CR.10 mice. Firstly, in regions of the frontal forebrain such as the CPu, Acb and cortex, C2CR.33 mice had significantly elevated 5-HT_{2C} receptor mRNA levels in all regions compared to WT mice (Figure 3.2a; effect of genotype $F_{1,45}$=151.56; p=<0.0001). In more posterior forebrain regions such as the cortex, dorsal hippocampus (CA1, CA2/3, DG), BLA and CP, there was a significant effect of the genotype of the mouse and its interaction with the regions tested (Figure 3.2b; $F_{5,60}$=15.84; p=<0.0001) with 5-HT_{2C} receptor mRNA levels elevated in all regions with the exception of the CP. Finally in ventral limbic regions such as the cortex, ventral hippocampus (CA1, CA2, CA3, DG) and the SN (Figure 3.2c), 5-HT_{2C} receptor mRNA levels were increased in the cortex and all regions of the ventral hippocampus in C2CR.33 mice compared to WT mice (Figure 3.2c; interaction of genotype and region $F_{5,42}$=6.15; p=0.0002) consistent with the increases described above. There was, however, no alteration in 5-HT_{2C} receptor mRNA levels in the SN of C2CR.33 mice.

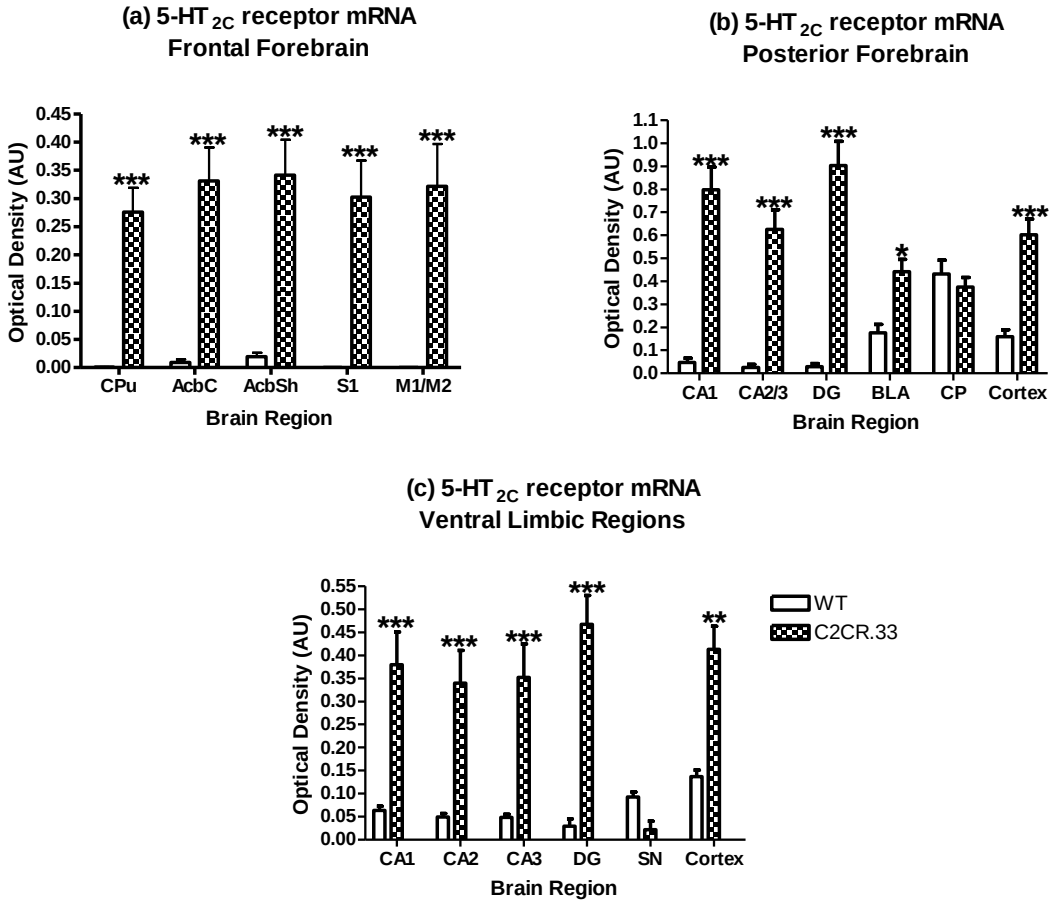


Figure 3.2: 5-HT$_{2C}$ receptor mRNA expression frontal to posterior in adult male C2CR.33 mice
5-HT$_{2C}$ receptor mRNA expression is increased in C2CR.33 mice compared to WT littermate control mice in (a) frontal forebrain, (b) posterior forebrain and (c) ventral limbic regions. Expression levels were measured by optical densitometry of *in situ* hybridisation autoradiographs. Data are expressed as mean±SEM in males backcrossed to C57BL/6 for 2 generations and analysed by unpaired, 2-way ANOVA with Bonferroni posttests; n= 6 per group. * p<0.05; ** p<0.01; *** p<0.001.

CPu = caudate putamen; AcbN = nucleus accumbens core; AcbSh = nucleus accumbens shell; S1 = primary somatosensory cortex; M1/M2 = primary/secondary motor cortex; CA1, CA2 or CA3 = CA1, CA2 or CA3 field of the hippocampus; DG = dentate gyrus; BLA = basolateral amygdaloid nucleus (anterior); CP = choroid plexus; SN = substantia nigra.

The transgene generated over-expression of 5-HT_{2C} receptor is higher in C2CR.33 mice than C2CR.10 mice (Table 1). Appetite and food intake is unaltered in these lines because 5-HT_{2C} receptor expression is unaltered in the hypothalamus, the centre for appetite regulation (Suzuki *et al*., 2010).

Table 3.1: Percentage increase in 5-HT_{2C} receptor mRNA levels in C2CR>10 or C2CR.33 mice compared to WT mice

C2CR.33 mice have higher % increase in 5-HT_{2C} receptor mRNA than C2CR.10 mice backcrossed to C57BL/6 for 2 generations. C2CR.10 and C2CR.33 mice compared to WT littermate control mice by two-way ANOVA with Bonferroni posttests.

Brain Region	C2CR.33	C2CR.10
Acb C	1770.9 ± 322.71	221.02 ± 43.5
Dorsal CA1	1688.09 ± 210.53	709.79 ± 80.66
Dorsal CA3	2376.64 ± 319.28	634.55 ± 67.62
Dorsal DG	3164.94 ± 369.52	20311.99 ± 1976.26
Ventral CA3	726.90 ± 149.25	191.49 ± 19.33

To determine if transgene expression results in a regulation of the endogenous receptor, hence altering the total levels and potentially the ratio of edited isoforms, the endogenous 5-HT_{2C} receptor was measured in all lines. The endogenous 5-HT_{2C} receptor mRNA expression in C2CR.10 and C2CR.33 mice was found to be unaltered by the elevated 5-HT_{2C} receptor expression resulting from the transgene with no effect when compared to WT mice (Figure 3.3; p=0.333).

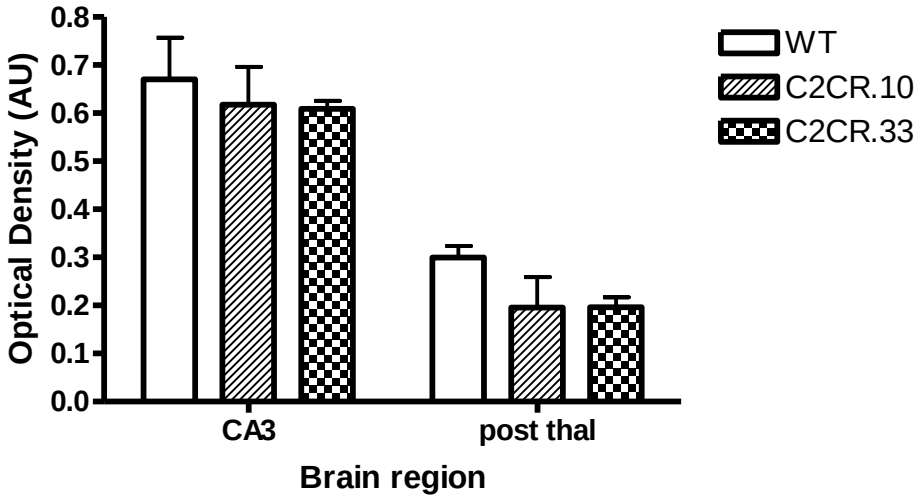


Figure 3.3: Endogenous 5-HT_{2C} receptor mRNA expression in C2CR.10 and C2CR.33 mice No alteration in the endogenous 5-HT_{2C} receptor mRNA expression is found in C2CR.10 or C2CR.33 mice compared to WT mice. Experiment carried out by A. Kimura; analysed by P. Stevenson. Expression levels were measured by optical densitometry of *in situ* hybridisation autoradiographs. Data are expressed as mean±SEM, in males backcrossed to C57BL/6 for 2 generations. C2CR.10 and C2CR.33 mice compared to WT littermate control mice by unpaired, 2-way ANOVA with Bonferroni posttests; n=2-3 per group.

CA3 = CA3 field of the ventral hippocampus; post thal = post thalamic regions.

3.2.2 5-HT_{2C} receptor binding sites were increased in C2CR.10 mice in the hindbrain and in C2CR.33 mice in the forebrain

It is vital to establish if the increase in 5-HT_{2C} receptor mRNA found by *in situ* hybridisation (section 3.3.1) results in increased protein activity. In order to assess this, 5-HT_{2C} receptor levels were measured using a ^{3}H-mesulergine (a ligand specific for 5-HT_{2C} receptor) binding assay in membrane preparations. C2CR.10 mice, backcrossed to C57BL/6 for 5 generations, showed no difference in binding compared to WT mice in the forebrain plus midbrain (Figure 3.4A; p=0.995). Unexpectedly, a significant increase in binding was found in the membranes

prepared from the hindbrain of C2CR.10 mice compared to WT mice (p=0.0006) which could indicate transgene expression in the cerebellum even though the CamK promoter is usually associated with a forebrain specific expression pattern. There was a two-fold increase in ^{3}H-mesulergine binding to membranes prepared from the forebrain of C2CR.33 mice (backcrossed to C57BL/6 for 4 generations) compared to WT mice (Figure 3.4b; p=0.045), indicating that the increased 5-HT_{2C} receptor mRNA level was translated into protein expressed at the cell surface or internal membranes. In membranes from the hindbrain, where the transgene is not expected to be expressed, ^{3}H-mesulergine binding was unaltered in C2CR.33 mice compared to WT mice (p=0.882).

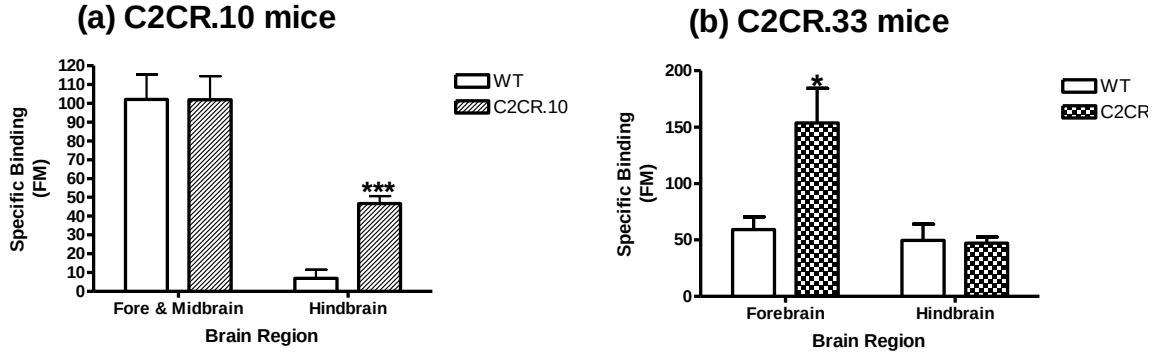


Figure 3.4: 5-HT_{2C} receptor number in adult male C2CR.10 and C2CR.33 mice determined by ^{3}H-mesulergine binding assay

5-HT_{2C} receptor number was unaltered in the forebrain and midbrain membrane preparations and increased in the hindbrain membrane preparation of C2CR.10 mice compared to WT mice (a). In forebrain membrane preparations from C2CR.33 mice, 5-HT_{2C} receptor number was increased compared to WT mice, with no alterations between genotypes in the hindbrain membrane preparation (b). C2CR.10 mice backcrossed to C57BL/6 for 5 generations and C2CR.33 mice backcrossed to C57BL/6 for 4 generations. Data are expressed as mean±SEM; C2CR.10 and C2CR.33 mice compared to WT littermate control mice for each region using unpaired Student's t test; n=4 per group. * p<0.05, *** p<0.001.

3.2.3 5-HT_{2C} receptor over-expression in C2CR.10 and C2CR.33 mice increases anxiety-like behaviour

The 5-HT_{2C} receptor has been implicated widely in the control of mood and in particular anxiety, with agonists reported to be anxiogenic while antagonists are anxiolytic (Chapter 1). In order to examine whether the over-expression of 5-HT_{2C} receptor in C2CR.10 and C2CR.33 mice affects anxiety-like behaviour, the elevated plus maze (EPM) and the open field (OF) tests were used. These are both well-established standard tests of anxiety-like behaviour in rodents and rely on the natural instinct of rodents to avoid brightly lit exposed regions of the mazes. In this set of experiments all mice used had been backcrossed to C57BL/6 for 3 generations.

In the EPM, anxiety-like behaviour is indicated by a decrease in exploration, time and/or distance travelled, in the elevated and exposed, light open arms in comparison to control animals. The OF works on a similar principle, with decreased time and/or distance travelled in the exposed, brightly lit central region of the maze indicating increased anxiety-like behaviour. Both of these measures (percentage time and percentage distance travelled) must be considered before a conclusion can be drawn for both the EPM and the OF. C2CR.33 mice were found to spend a similar percentage of time in the open arms of the EPM compared to WT mice (Figure 3.5a, $p=0.225$). In the OF, C2CR.33 mice spent significantly less time in the inner zone, compared to WT mice (Figure 3.5b, $p=0.031$), indicating an increase in anxiety-like behaviour.

Some mice may freeze when they enter the exposed regions and so although their percentage time would not show significance, their percentage distance would. This was found to be the case in C2CR.33 mice in the EPM with the percentage distance travelled within the open arms significantly reduced compared to WT mice (Figure 3.5c, $p=0.024$). This indicates that although C2CR.33 mice spend the same percentage of time in the open arms compared to WT mice, they travel a significantly lower distance in these regions compared to controls, indicating that they have frozen due to increased anxiety. In the OF C2CR.33 mice show no

alteration in the percentage of distance travelled in the exposed zone compared to WT mice (Figure 3.5d, p=0.483).

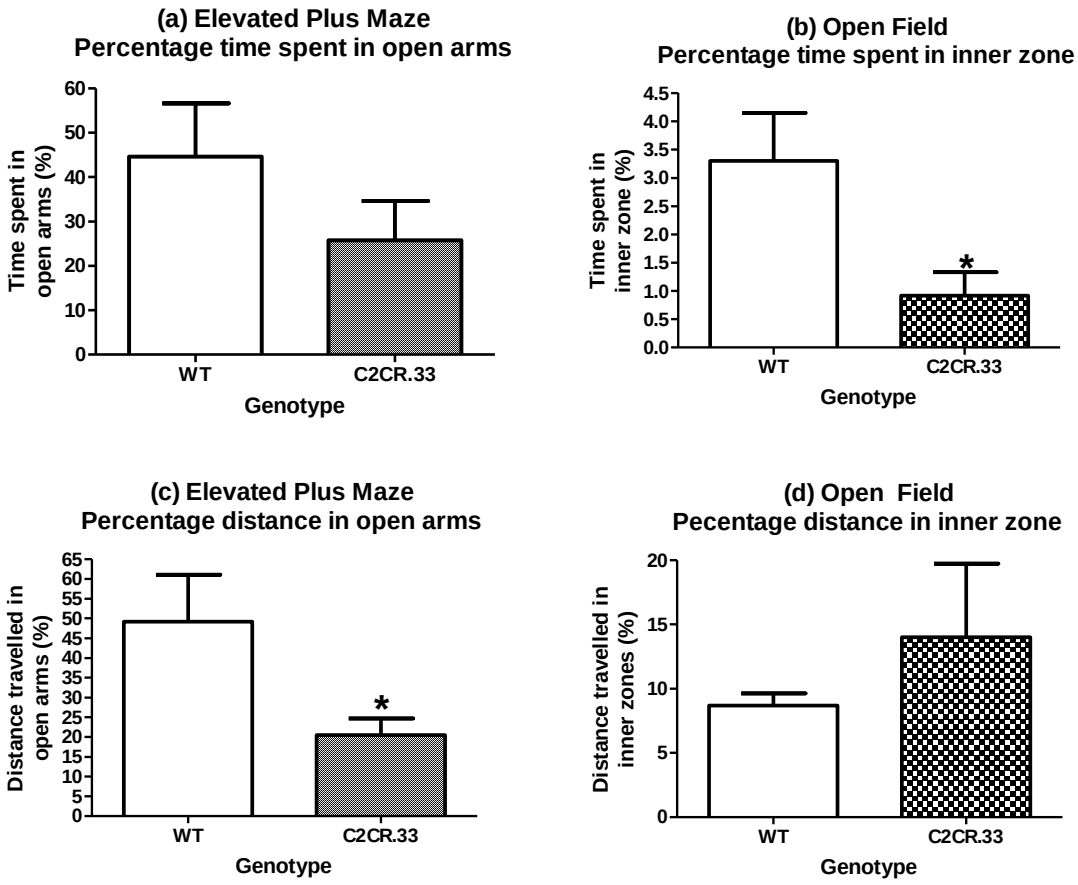


Figure 3.5: Behaviour in the elevated plus maze and open field test in male C2CR.33 mice

No alterations were found in the time spent (a) in the open arms of the EPM between genotypes. The percentage of time spent in the inner zones of the OF (b) was reduced in C2CR.33 mice compared to WT mice. The percentage of distance travelled in the open arms of the EPM (c) was reduced in C2CR.33 mice compared to WT controls. No alterations between genotypes were found in the percentage of distance travelled in the inner zones of the OF (d). All mice were males backcrossed to C57BL/6 for 3 generations. Data are expressed as mean±SEM; generated by A. Kimura. C2CR.33 mice compared to WT littermate control mice by unpaired Student's t test; EPM n =9-11 per group and OF n =5-6 per group * p <0.05.

Compared to WT, C2CR.10 mice showed no alterations in their behaviour in the EPM in the distance travelled in the open arms (% distance in open arms WT = 54.27 ±8; C2CR.10 = 53.38 ±8.5). In the OF, C2CR.10 mice showed a trend to spend less time (Figure 3.6a; p= 0.089) and fewer crossings within the inner zone (Figure 3.6b; p= 0.068), compared to WT mice.

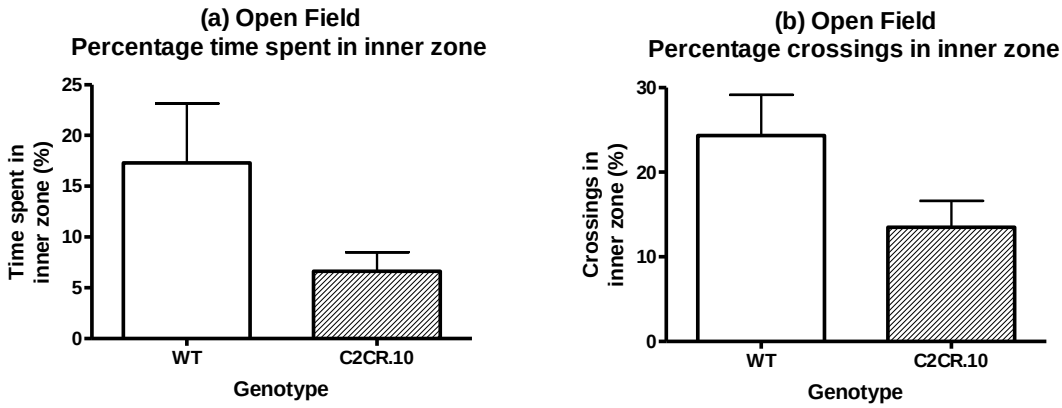


Figure 3.6: Behaviour in the open field in C2CR.10 mice
C2CR.10 mice show a trend to spend less time (a) and fewer crossings (b) within the inner zone of the OF compared to WT mice. All mice were backcrossed to C57BL/6 for 3 generations. Data are expressed as mean±SEM. C2CR.10 mice were compared to WT littermate control mice by unpaired Student's t test; n=6 per group.

Total locomotion during the EPM and OF tests was measured to determine if locomotion in a novel environment with a component of stress was altered by over-expression of 5-HT_{2C} receptor in the forebrain. In the EPM, C2CR.33 mice travelled a similar total distance during the test compared to WT mice (Figure 3.7a, p=0.504), but they travelled significantly less than WT mice in the OF (Figure 3.7b, p=0.027), indicating hypolocomotion in response to a novel environment. No hypolocomotor phenotype was found in C2CR.10 mice compared to WT mice (total distance travelled in EPM WT mice p=320.25 ±39; C2CR.10 mice p=369.66 ±52).

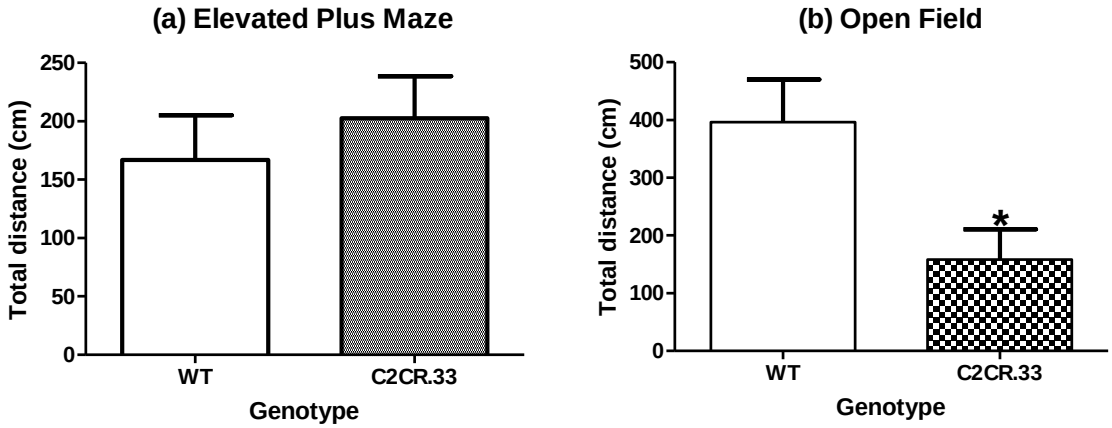


Figure 3.7: Distance travelled in the elevated plus maze and open field in male C2CR.33 mice

The total distance travelled in the EPM (a) was unaltered between genotypes but was reduced in C2CR.33 in the OF (b). All mice were males backcrossed to C57BL/6 for 3 generations. Data are expressed as mean±SEM,. C2CR.33 mice compared to WT littermate control mice by unpaired Student's t test; EPM n=9-11per group and OF n=5-6 per group. * p<0.05.

In summary, C2CR.33 mice show a slight anxiety phenotype, in both the EPM and the OF, and hypolocomotion in the novel environment with a component of stress (OF only) compared to WT mice, while C2CR.10 mice only show a very slight anxiety phenotype in the OF and no locomotor phenotype.

3.2.4 5-HT_{2C} receptor over-expression causes hypolocomotion in C2CR.10 and C2CR.33 mice

5-HT_{2C} receptor transmission has been implicated in activity/locomotion, with agonists, such as mCPP causing hypolocomotion (Gleason *et al.*, 2001). As C2CR.33 mice travelled less distance in the OF compared to WT mice, voluntary wheel running was tested in these mice to investigate their general activity. Voluntary wheel running allows the locomotion of an individual mouse to be monitored over an indefinite period of time in their home cage, providing data on the total activity as well as the circadian pattern of wheel running throughout the experiment. In C2CR.10 mice wheel running activity was significantly decreased compared to WT mice, entirely due to reduced activity in the dark phase (Figure 3.8a; interaction $F_{2,30}=7.62$; $p=0.002$; effect of genotype $F_{1,30}=35.84$; $p<0.0001$). However, C2CR.33 mice showed no alterations compared to WT mice in wheel running behaviour (Figure 3.8b; interaction $F_{2,30}=1.14$; $p=0.333$; genotype $F_{1,30}=0.15$; $p=0.703$).

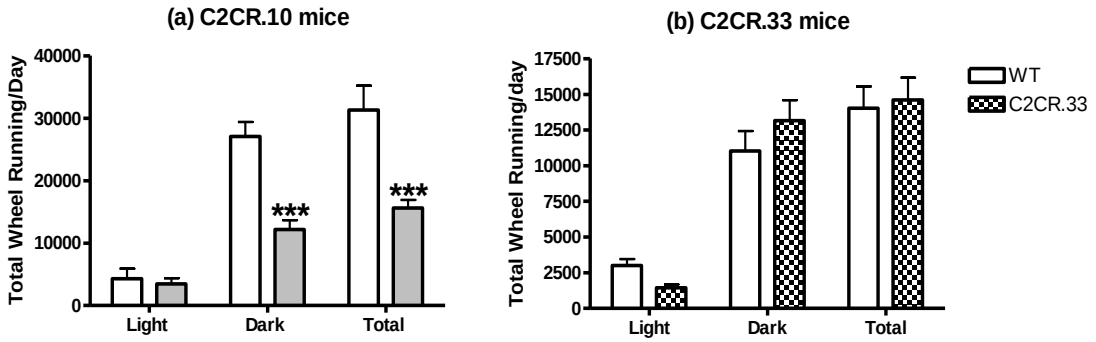


Figure 3.8: Voluntary wheel running in male C2CR.10 and C2CR.33 mice
Wheel running was reduced in C2CR.10 mice (a) in the dark phase compared to WT mice. No alteration in wheel running was found between C2CR.33 mice and WT mice (a). All mice were individually housed males, backcrossed to C57BL/6 for 3 generations and C2CR.33 data generated by A.Kimura. Data are expressed as mean±SEM; C2CR.10 or C2CR.33 mice compared to WT littermate control mice by two-way ANOVA with Bonferroni posttests or unpaired Student's t test; n=6 per group. *** $p<0.001$.

3.3 Characterisation of models during backcrossing onto C57BL/6

In the process of backcrossing the C2CR lines to C57BL/6, the phenotype of different generations was tested. This was to check if the modest increase in anxiety-like behaviour and hypolocomotion seen in these mice at early backcrosses, would remain constant as genetic background can exert a strong influence on behaviour and the C2CR lines were on a mixed genetic background (CBA, C3H and C57BL/6).

3.3.1 The increase in anxiety-like behaviour was lost in C2CR.33 mice during backcrossing onto C57BL/6

The EPM and OF, tests of anxiety-like behaviour, were carried out over different generations during the backcrossing process; at generations 4, 6 and 7 on mice kept in a secluded quiet room. As before, both the percentage of time spent and distance

travelled in the exposed regions of the maze were used to assess anxiety-like behaviour. Generations 6 and 7 of C2CR.33 mice showed no alteration from WT mice in the percentage of time spent in the open arms of the EPM (Table 2; interaction $F_{1,35}$=0.97; p=0.332). This was consistent with the initial experiments carried out in this line in the EPM. In the OF, in the initial characterisation using generation 3, a significant reduction in the percentage of time spent in the inner zone by C2CR.33 mice compared to WT mice was found (see Figure 3.5b). However, following backcrossing to C57BL/6 for 4, 6 and 7 generations there was no alteration from WT mice in the percentage of time spent in the inner zone of the OF in (Table 2; interaction $F_{2,64}$=0.44; p=0.648).

Table 3.2: Percentage time (of total time) spent in the open arms of the elevated plus maze or inner zone of the open field following backcrossing onto C57BL/6 in male C2CR.33 mice

No alterations between genotypes found in the percentage time spent in the open arms of the EPM or inner zone of the OF in male mice backcrossed to C57BL/6 for 4, 6 or 7 generations. Data expressed as mean±SEM; C2CR.33 mice were compared to WT littermate control mice by two-way ANOVA with Bonferroni posttests; n=8-14 per group.

	Generation 4		Generation 6		Generation 7	
	WT	C2CR.33	WT	C2CR.33	WT	C2CR.33
EPM			28.6 ±5.4	24.19 ±5.63	20.8±2.14	24.06±3.16
OF	18.6±5.71	17.0±4.43	17.4±3.04	13.2±2.67	21.7±4.77	21.1±3.08

In the initial characterisation of C2CR.33 mice backcrossed to C57BL/6 for 3 generations, although C2CR.33 mice had a similar percentage of time spent in the open arms compared to WT mice in the EPM, they travelled a significantly lower percentage of their total distance in the open arms compared to WT mice. It was concluded that C2CR.33 mice were freezing in the open arms due to increased anxiety-like behaviour. Following backcrossing to C57BL/6 for 6 and 7 generations, C2CR.33 mice did not differ from WT mice in the percentage of their total distance they travelled in the open arms in the EPM (Table 3; $F_{1,33}$=0.00; p=0.959). In the

OF, generations 4, 6 and 7 of C2CR.33 mice did not differ from WT mice in the percentage of distance travelled in the inner zone of the OF (Table 3; $F_{2,62}$=0.89; p=0.415), consistent with the data from generation 3 mice.

Table 3.3: Percentage distance (of total distance) travelled in the open arms of the elevated plus maze or inner zone of the open field following backcrossing onto C57BL/6 in male C2CR.33 mice

No alterations between genotypes found in the percentage distance travelled in the open arms of the EPM or inner zone of the OF in male mice backcrossed to C57BL/6 for 4, 6 or 7 generations. Data expressed as mean±SEM; C2CR.33 mice were compared to WT littermate control mice by two-way ANOVA with Bonferroni posttests; n=8-14 per group.

	Generation 4		Generation 6		Generation 7	
	WT	C2CR.33	WT	C2CR.33	WT	C2CR.33
EPM			23.1±4.3	24.3±4.94	29.53±4.04	31.2±3.85
OF	25.5±3.56	31±3.81	17.2±2.56	17.1±3.05	21.7±3.95	19.8±1.95

Female C2CR.33 mice (backcrossed to C57BL/6 for 7 generations) were also tested for anxiety-like behaviour. However, in the EPM, no alteration from WT mice was found in either the percentage of time spent (Table 4; p=0.825) or percentage of distance travelled in the open arms (Table 4; p=0.473) or in the percentage of time spent (Table 4; p=0.832) or percentage of distance travelled in the inner zone (Table 4; p=0.471) of the OF. In a gender comparison of C2CR.33 male and female mice, backcrossed to C57BL/6 for 7 generations, females have a higher percentage of distance travelled in the open arms of the EPM (Figure 3.9; $F_{1,34}$=55.00; p<0.0001) but no other ethiological parameters were changed.

Table 3.4: Behaviour in the elevated plus maze and open field test in female C2CR.33 mice

No alterations between genotypes found in the percentage of time spent or distance travelled in the open arms of the EPM or inner zone of the OF in female mice backcrossed to C57BL/6 7 generations. Data expressed as mean±SEM, with percentages representing the percentage of total distance or time throughout test; C2CR.33 mice were compared to WT littermate control mice by two-way ANOVA with Bonferroni posttests; n=7-9 per group.

	% Time EPM	% Distance EPM	% Time OF	% Distance OF
WT	37.07 ± 5.13	62.72 ± 5.49	20.25 ± 3.84	23.64 ± 4.22
C2CR.33	35.17 ± 6.92	68.78 ± 6.11	19.11 ± 3.37	19.63 ± 2.80

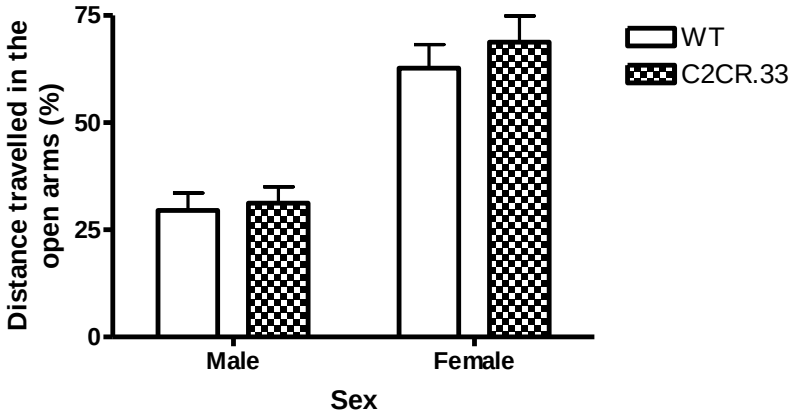


Figure 3.9: Distance in the open arms of the elevated plus maze in C2CR.33 males and female mice

The percentage of distance travelled in the open arms of the EPM was similar between genotypes with female mice having increased percentage distance travelled compared to male mice. Mice were males and females backcrossed to C57BL/6 for 7 generations. Data are expressed as mean ±SEM, n=7-12, C2CR.33 compared to WT littermate control mice by two-way ANOVA with Bonferroni posttests.

During the initial characterisation of C2CR.33 mice (backcrossed to C57BL/6 for 3 generations) although no alteration in locomotion was found in C2CR.33 mice compared to WT mice in the EPM, a significant reduction in locomotion was found in the OF. In C2CR.33 mice, backcrossed to C57BL/6 for 6 ($p=0.232$) and 7 generations ($p=0.980$) the total distance travelled in the EPM throughout the test was similar between genotypes (Figure 3.10a), consistent with the previous results. There was an effect of the generation of backcrossing onto C57BL/6 with generation 7 mice travelling a greater distance around the maze than generation 6 (Figure 3.9a; $F_{1,33}=29.03$; $p<0.0001$ by two-way ANOVA). The hypolocomotion found in C2CR.33 mice in the OF during the initial characterisation remained following 4 generations of backcrossing onto C57BL/6 (Figure 3.10b; $p=0.021$) but was not seen after 6 (Figure 3.10b; $p=0.52$) or 7 generations (Figure 3.9b; $p=0.334$), there was a significant effect of generation ($F_{1,58}=10.77$; $p=0.0001$, two-way ANOVA) and interaction with genotype ($F_{1,58}=3.08$; $p=0.05$, two-way ANOVA). The loss of hypolocomotion in C2CR.33 mice actually appeared due to a reduction in locomotion across generations of WT mice (Figure 3.10c; $p<0.0001$, one-way ANOVA), while the locomotion of C2CR.33 mice was unaltered across generations (Figure 3.10d; $p=0.459$, one-way ANOVA).

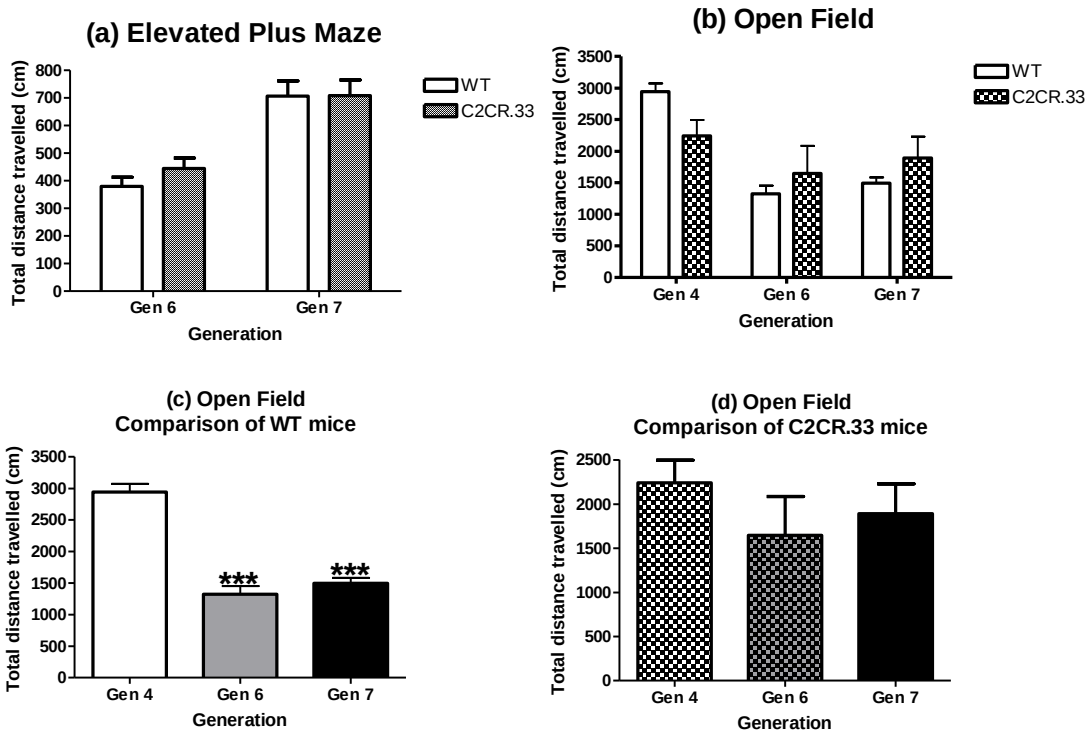


Figure 3.10: Total distance travelled in the elevated plus maze or open field in male C2CR.33 mice backcrossed to C57BL/6 for 4, 6 or 7 generations
The total distance travelled in the EPM was unaltered between genotypes (a) although mice backcrossed to C57BL/6 for 7 generations travelled a greater distance than those backcrossed for 6 generations. In the OF the total distance travelled (b) was reduced in C2CR.33 mice compared to WT mice following backcrossing to C57BL/6 for 4 generations. However, following 6 and 7 generations of backcrossing, no difference in locomotion was found between genotypes. Locomotion in the OF was reduced in WT mice (c) following 6 and 7 generations of backcrossing onto C57BL/6. Distance travelled in the OF by C2CR.33 mice (d) was unaltered by backcrossing. All mice were male. Data are expressed as mean±SEM; C2CR.33 mice were compared to WT littermate control mice by unpaired Student's t test, one-way ANOVA and/or two-way ANOVA with Bonferroni posttests; n=8-9 per group. *** $p<0.001$ for gen 4 vs gen 6 and gen 4 vs gen 7 in c.

Because the initial characterisation showed a difference in locomotion, C2CR.33 mice backcrossed to C57BL/6 for 6 generations, were tested in the OF for 30 minutes. Generation 6 mice were the earliest generation during backcrossing when no alteration was found between basal locomotion in this test. There was no difference between genotypes even in this 30 min test (Figure 3.11; $p=0.997$).

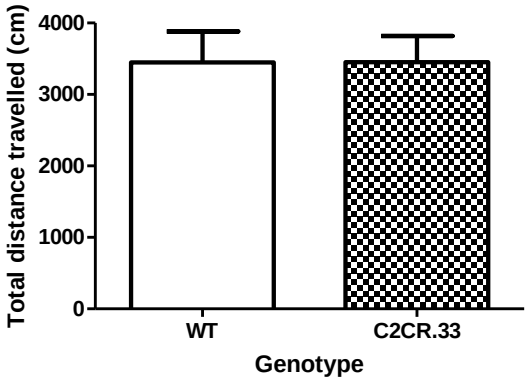


Figure 3.11: Total distance travelled during a 30 minute open field test in male C2CR.33 mice
Total distance travelled in the OF for 30 minutes is unaltered between genotypes. All mice were males backcrossed to C57BL/6 for 6 generations. Data are expressed as mean±SEM; C2CR.33 mice were compared to WT littermate control mice by unpaired Student's t tests; n=8-9 per group.

Following backcrossing, C2CR.33 female mice were also tested in both the EPM and OF to determine if locomotion was altered. However, they did not differ from WT mice in either the EPM (Table 5; p=0.234, unpaired Student's t test) or OF (p=0.505, unpaired Student's t test). In the EPM a significant effect of gender was found ($F_{1,34}$=17.61; p=0.0002, two-way ANOVA) with female mice travelling a greater distance throughout the test than male mice.

Table 5: Total distance travelled in the elevated plus maze and open field in C2CR.33 mice backcrossed to C57BL/6 for 7 generations

Female C2CR.33 mice did not differ from WT control mice in total distance travelled in the EPM or OF. All mice were backcrossed to C57BL/6 for 7 generations. Data are expressed as mean±SEM; C2CR.33 mice were compared to WT littermate control mice by unpaired Student's t tests; n=7-9.

	Total Distance EPM	Total Distance OF
WT	904.72 ± 54.16	1901.15 ± 185.15
C2CR.33	1009.23 ± 64.74	2084.27 ± 187.32

In summary, the weak anxiety phenotype that had been found in both the EPM and OF and the hypolocomotion found in the OF during the initial characterisation of C2CR.33 mice was lost during the backcrossing process onto C57BL/6.

3.3.2 5-HT_{2C} receptor over-expression in C2CR.33 mice did not alter fear responses or depressive-like behaviour in this study

The 5-HT_{2C} receptor is implicated strongly in depression, although the literature is conflicting with both agonists and antagonists of this receptor having antidepressant properties (Dunlop *et al*., 2006; Millan *et al*., 2006; Dekeyne *et al*., 2008). Activation of the 5-HT_{2C} receptor is involved in the regulation of the hypothalamic-pituitary-adrenal (HPA) axis through corticotrophin-releasing hormone (CRH). 5-HT_{2C} receptor is also implicated in fear responses due to altered influence on HPA axis regulation. Although the anxiety-like phenotype previously found in C2CR.10 and C2CR.33 mice was abolished following backcrossing onto C57BL/6, it is possible that other aspects of mood could be altered such as depressive-like behaviour. Hence, further tests were carried out. The conditioned avoidance test uses memory formation to an aversive stimulus as a measure of fear, with an increase in latency to move into the dark chamber following a foot shock indicating an increased

association and fear response (described in Chapter 2 section 2.3.5). C2CR.33 mice, backcrossed to C57BL/6 for 6 generations, showed no difference to WT mice in the time delay to enter the dark compartment (Figure 3.12a, p=0.149).

In order to test if there was a difference in depressive-like behaviour, a tail suspension test was used as described in Chapter 2 (2.3.4). Increased depressive-like behaviour is indicated by an increase in the percentage of time spent hanging passively during the test. C2CR.33 mice, backcrossed to C57BL/6 for 5 (Figure 3.12b; p=0.583) and 6 generations (Figure 3.12b; p=0.159), showed no tendency toward an alteration in depressive-like behaviour compared to WT mice.

These results suggest that over-expression of 5-HT_{2C} receptors in the forebrain does not cause either an increased fear response or depressive-like behaviour.

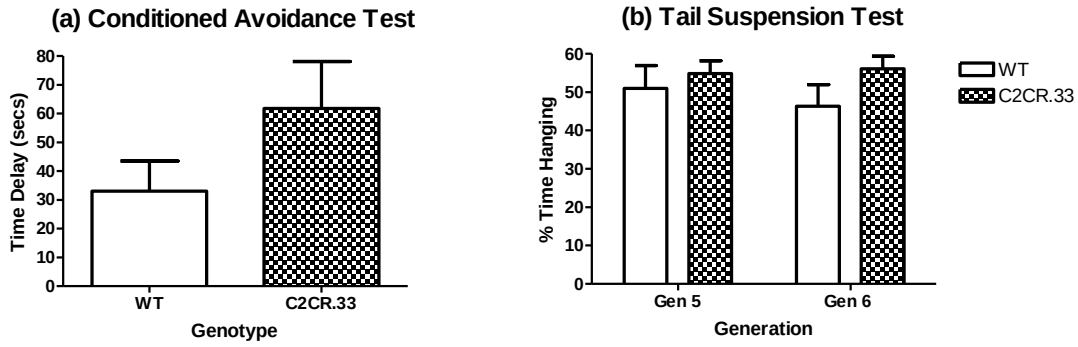


Figure 3.12: Behaviour during conditioned avoidance and tail suspension tests in male C2CR.33 mice
No difference between genotypes was found in the delay in time taken to enter the dark compartment between the conditioned avoidance test when a shock is given and the second repeat test in male mice backcrossed to C57BL/6 for 6 generations (a; n=20). The percentage of time spent passively hanging throughout the tail suspension test was similar in both genotypes in male mice backcrossed to C57BL/6 for 5 and 6 generations (B; n=9-11). Data are expressed as mean±SEM; C2CR.33 mice were compared to WT littermate control mice by unpaired Student's t test.

3.3.3 Following backcrossing onto C57BL/6, locomotion was unaltered in C2CR.10 mice

For the same reasons that anxiety-like behaviour was re examined during backcrossing, locomotion was also re-tested in C2CR.10 mice. During the initial characterisation C2CR.10 mice (backcrossed to C57BL/6 for 3 generations) showed hypolocomotion during the dark phase as well as reduced total daily running compared to WT mice. Following backcrossing to C57BL/6, there was no change in total wheel running, Figure 3.13; p=0.228) or in the light phase or dark phase (Figure 3.13; effect of genotype $F_{1,20}$=1.73; p=204).

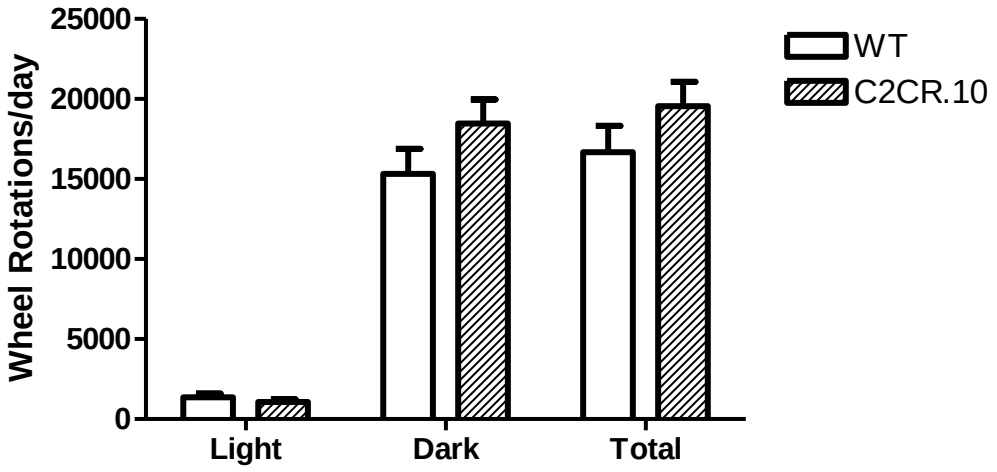


Figure 3.13: Voluntary wheel running behaviour in male C2CR.10 mice backcrossed to C57BL/6 for 4 generations
No difference between genotypes was found in basal wheel running in individually housed male mice backcrossed to C57BL/6 for 4 generations in normal lighting conditions. Data are expressed as mean±SEM; C2CR.10 were compared to WT littermate control mice by unpaired Student's t test or two-way ANOVA with Bonferroni posttests; n=6 per group.

3.3.4 Overexpression of 5-HT_{2C} receptors in C2CR.33 mice did not alter general agility and balance

In order to assess locomotion in more detail, C2CR.33 were subjected to a rotorod test to test their general agility and balance. This was carried out to check that the presence of the transgene was not impairing their physical ability.

C2CR.33 mice, backcrossed to C57BL/6 for 6 or 7 generations, performed just as well as the WT littermate control mice on the rotorod at 6 months of age (Figure 3.14a; $F_{1,28}$=0.14; p=0.714). An effect of generation was found, with generation 7 mice having reduced agility and balance compared to generation 6 mice (Figure 3.14a; $F_{1,28}$=29.68; p<0.0001), although no interaction of genotype and generation was found ($F_{1,28}$=2.36; p=0.136). C2CR.33 mice, backcrossed to C57BL/6 for 6 generations, also had similar general agility and balance on the rotorod as WT mice at 3 months of age (data not shown, p=0.133). A comparison of C2CR.33 male and female mice showed a significant reduction in agility and balance in both genders compared to WT mice (Figure 3.14b, $F_{1,28}$=4.92; p=0.035). Female mice had increased general agility and balance compared to males ($F_{1,28}$=10.59; p=0.003) but there was no interaction of genotype with sex ($F_{1,28}$=0.03; p=0.854).

In summary, increased 5-HT_{2C} receptor in the forebrain does not affect general agility and balance in C2CR.33 male or female mice, although female mice had increased agility and balance than male mice on the whole.

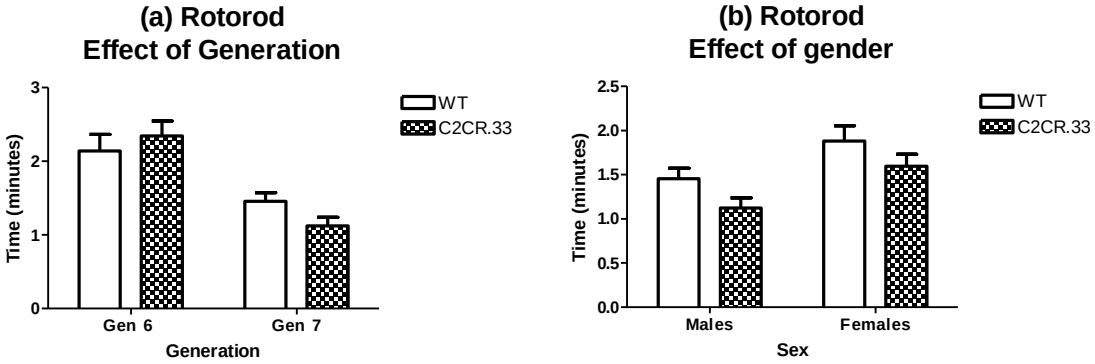


Figure 3.14: Effect of generation and gender during the rotorod test in C2CR.33 mice There was no effect of genotype on the length of time male mice were able to stay on the rotorod (a) without circling or falling off, although generation 7 mice were not as agile as generation 6 male mice. Male mice (a) were backcrossed to C57BL/6 for 6 and 7 generations, age 6 months, n=16-18 per group. Male and female C2CR.33 mice were had reduced agility and balance than WT mice (b) and female mice had an increased length of time on the rotorod compared to male mice. Male and female mice (b) were backcrossed to C57BL/6 for 7 generations, age 6 months, n=12-16 per group. Data are expressed as mean±SEM; C2CR.33 mice were compared to WT littermate control mice by two-way ANOVA with Bonferroni posttests.

3.3.5 The pattern of 5-HT_{2C} receptor mRNA over-expression in C2CR.33 mice was unaffected by backcrossing to C57BL/6

It can be concluded from the behavioural assessment of the lines that the weak hypolocomotion and anxiety-like behavioural phenotypes found in the initial characterisation were lost during backcrossing to C57BL/6. There are many possible reasons for this that must be investigated in order to determine the underlying cause of this. One major possibility sometimes observed in transgenic lines is transgene silencing. Accordingly, 5-HT_{2C} receptor mRNA and binding levels were re-assessed in generation 7 and 6 mice respectively.

Following 7 generations of backcrossing to C57BL/6, 5-HT_{2C} receptor mRNA levels remained elevated in C2CR.33 mice in the same brain regions (the CPu, AcbC, AcbSh, primary somatosensory cortex, primary and secondary motor cortex, BLA, cortex and dorsal and ventral CA1, CA2, CA3 fields of the hippocampus and DG) found in C2CR.33 mice backcrossed to C57BL/6 for 2 generations (Figure 3.15 compare with Figure 3.3 from generation 2 mice). The exception to the identical pattern between the two C2CR.33 generations is that generation 7 mice had increased 5-HT_{2C} receptor compared to WT mice, which shows no alteration in generation 2 mice.

Additional regions to those examined in generation 2 mice were examined in C2CR.33 mice backcrossed to C57BL/6 for 7 generations. Compared to WT mice, no alterations were found in the paraventricular nucleus (PVN), thalamus, SM, raphe nulceus or pontine, although a significant increase in 5-HT_{2C} receptor mRNA level was found in the SUBC of C2CR.33 mice (Figure 3.15).

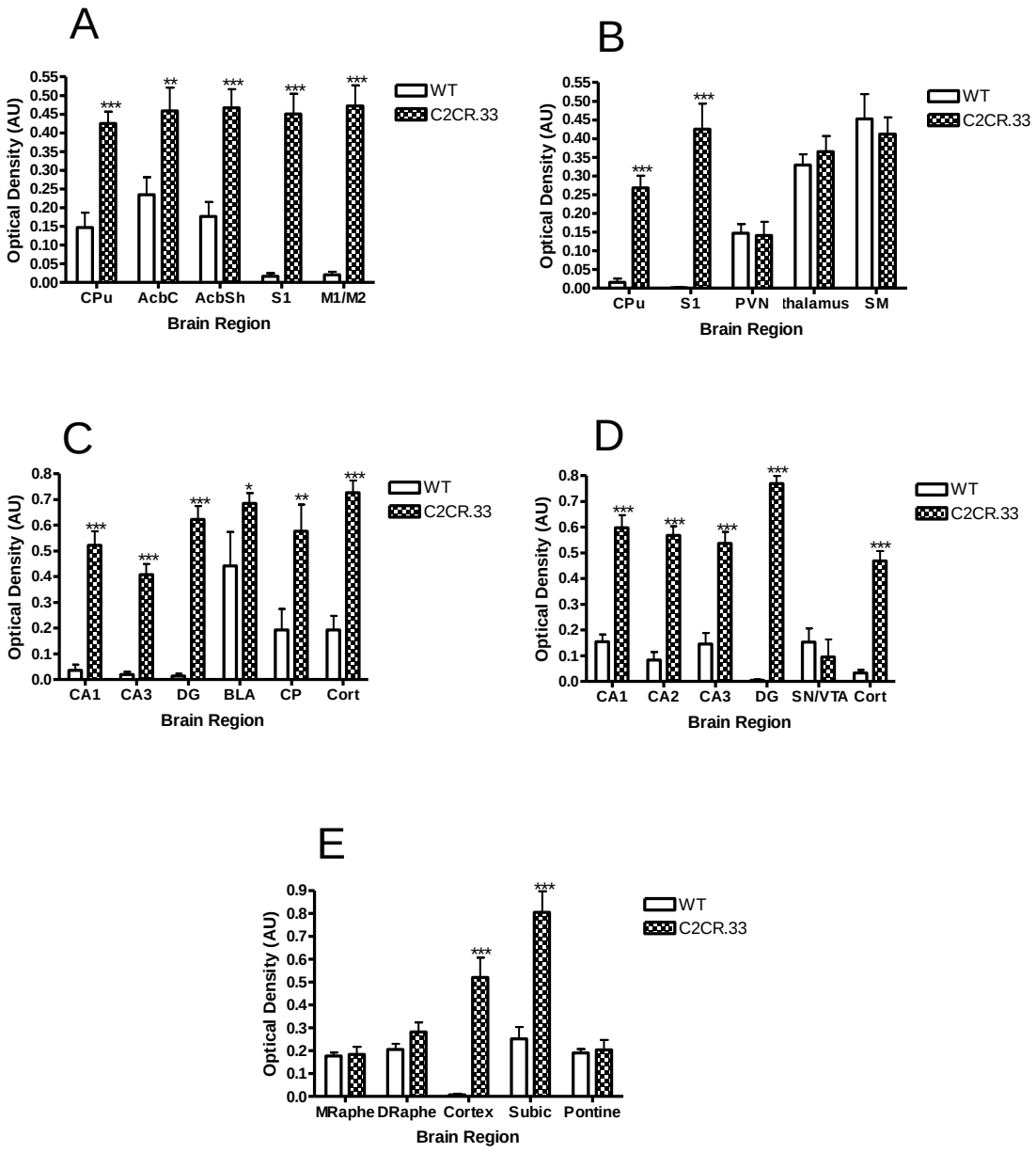


Figure 3.15: 5-HT_{2C} receptor mRNA expression frontal to posterior in adult male C2CR.33 mice

5-HT_{2C} receptor mRNA expression increase is maintained in C2CR.33 mice compared to WT littermate control mice in (a) Striatal (b) PVN (c) frontal forebrain, (d) posterior forebrain, (e) ventral limbic regions and (f) raphe nulcei. Expression levels were measured by optical densitometry of *in situ* hybridisation autoradiographs. Data are expressed as mean±SEM in males backcrossed to C57BL/6 for 7 generations and analysed by unpaired, 2-way ANOVA with Bonferroni posttests; n= 6 per group. * p=<0.05; ** p=<0.01; *** p=<0.001. Interaction of genotype and region is (a) p=0.0331, (b) p=<0.0001, (c) p=0.0528, (d) p=<0.0001, (e) p=<0.0001

CPu = caudate putamen; AcbN = nucleus accumbens core; AcbSh = nucleus accumbens shell; S1 = primary somatosensory cortex; M1/M2 = primary/secondary motor cortex; PVN = paraventricular nucleus, CA1, CA2 or CA3 = CA1, CA2 or CA3 field of the hippocampus; DG = dentate gyrus; BLA = basolateral amygdaloid nucleus (anterior); CP = choroid plexus; SN = substantia nigra; CORT = cortex MRaphe = median raphe nucleus; DRaphe = dorsal raphe nucleus.

In order to verify that the elevated mRNA levels were still being translated to protein, 5-HT_{2C} receptors were measured again using a ^{3}H-mesulergine binding assay. A two-fold increase in ^{3}H-mesulergine binding was found in the forebrain of C2CR.33 mice backcrossed to C57BL/6 for 7 generations (Figure 3.16, unpaired Student's t test p=0.012) indicating increased 5-HT_{2C} receptor number . Similarly to the initial characterisation in C2CR.33 mice, no alteration in ^{3}H-mesulergine binding levels were found in hindbrain membrane preparations (p=0.657).

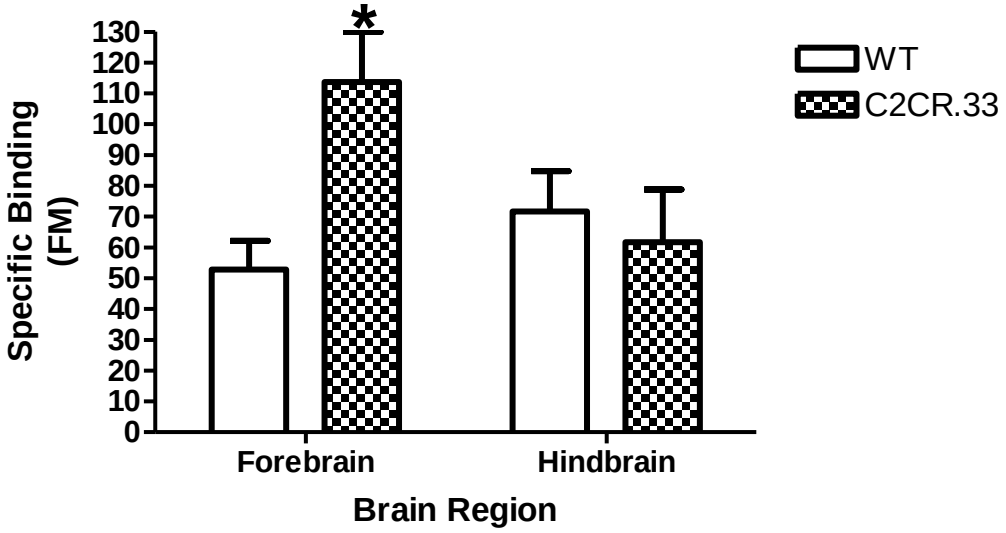


Figure 3.16: 5-HT_{2C} receptor number in adult male C2CR.33 mice determined by ^{3}H-mesulergine binding assay
In forebrain membrane preparations from C2CR.33 mice, 5-HT_{2C} receptor number was increased compared to WT mice, with no alterations between genotypes in the hindbrain membrane preparation. C2CR.33 mice backcrossed to C57BL/6 for 7 generations. Data are expressed as mean±SEM; C2CR.33 mice compared to WT littermate control mice for each region using unpaired Student's t test; n=6 per group. * p<0.05

3.4 Response to 5-HT_{2C} receptor ligands in C2CR.10 and C2CR.33 mice

When these experiments were undertaken the aim was to examine if the hypolocomotor effects of transgene expression could be due to an elevated forebrain 5-HT_{2C} receptor number, an increased ligand sensitivity of transgene encoded 5-HT_{2C} receptor (the unedited isoform has a higher constitutive activity and an increased ligand sensitivity *in vitro*) or a combination of both. However, even following the loss of behavioural phenotype during backcrossing onto C57BL/6 experiments where ligands were administered were continued in case this might uncover a phenotype.

The behavioural response to 5-HT_{2C} receptor ligands, agonists and antagonists, were determined. Increased 5-HT_{2C} receptors in C2CR.33 and C2CR.10 mice may cause a response at a much lower dose of agonist compared to WT mice or alternatively no effect on Kd might be predicted but a bigger response in C2CR lines at a given dose might be found. On the other hand, due to the over-expressed receptor being fully unedited it is possible that all of these receptors may be constitutively active and so no further response in behaviour may be found upon the injection of agonist. Inverse agonists are a useful tool to elucidate whether constitutive activity is present in a model. Ligands were administered by intraperitoneal injection 30 minutes prior to testing in the OF or 30 minutes prior to the onset of the dark phase in voluntary wheel running cages.

3.4.1 The effect of agonists and antagonists on behaviour in novel environment

The open field test was used to assess the acute effect of 5-HT_{2C} receptor agonists on both locomotor activity in a novel environment and anxiety-like behaviour. The mixed agonist, mCPP, which has been shown to induce anxiety and cause hypolocomotion but is preferential for the 5-HT_{2C} receptor (Hackler *et* al., 2007; Stiedle *et al*., 2007), was administered. Previously, Dr A. Kimura found a weak anxiety-like phenotype in C2CR.33 mice backcrossed to C57BL/6 for 3 generations, with a reduction in the percentage time spent in the inner zone of the OF (Figure 3.17a) following vehicle injection. The significant reduction on the percentage of time spent in the inner zone was not seen following the administration of mCPP, due to a high level of variation in the WT mice group (Figure 3.17a, interaction between genotype and drug $F_{1,15}$=0.00; p=0.993), and administration of 0.3mg/kg mCPP had no effect on the percentage of time spent in the inner zone in either C2CR.33 or WT mice ($F_{1,15}$=0.11; p=0.747). However, a dose of 0.3mg/kg mCPP significantly reduced the percentage of distance travelled by C2CR.33 mice in the inner zone compared to WT mice (Figure 3.17b; interaction of genotype and drug $F_{1,16}$=6.87; p=0.019). This indicates that C2CR.33 mice have a higher sensitivity to the anxiolytic effect of mCPP than WT mice. A strong hypolocomoter phenotype had been found in C2CR.33 mice, backcrossed to C57BL/6 for 3 generations, compared to WT mice in the OF (Figure 3.17c). Administration of mCPP to WT mice significantly decreased total distance travelled in the OF, but was unable to further reduce locomotion in C2CR.33 mice (Figure 3.17c, interaction of genotype and drug $F_{1,16}$=10.32; p=0.005). This suggests that 5-HT_{2C} receptor over-expression is maximally inhibiting locomotion under basal conditions. Due to the lack of effect of mCPP (compared to saline injection) on the total distance travelled it can be concluded that the increased anxiety-like behaviour following mCPP administration (suggested by the reduced percentage distance travelled in the inner zone) was not attributable to a sedative effect of mCPP.

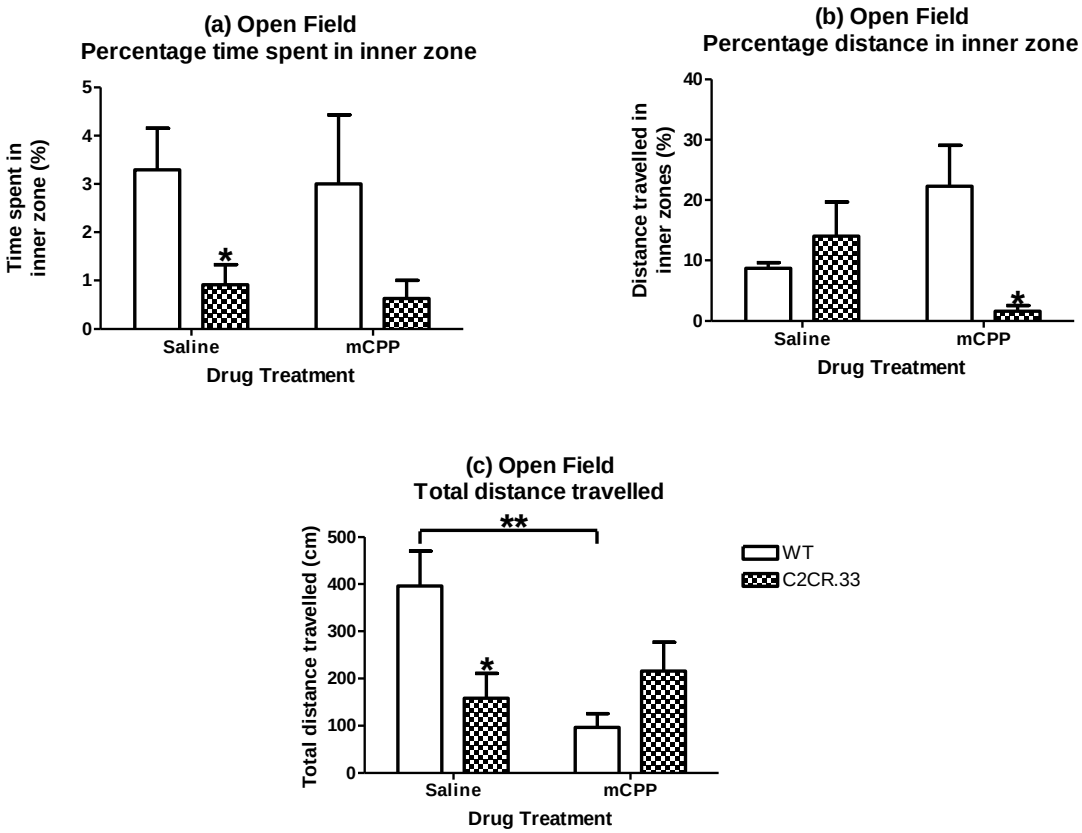


Figure 3.17: Effect of mCPP on behaviour in the open field in male C2CR.33 mice
0.3mg/kg mCPP given in the OF did not alter the percentage of time spent in the inner zone (a) in C2CR.33 or WT mice, reduced the percentage of distance travelled in the inner zone (b) in C2CR.33 mice but not in WT mice and reduced the total distance travelled (c) in WT mice but was unable to further reduce locomotion in C2CR.33 mice. All mice were males, backcrossed to C57BL/6 for 3 generations. Data are expressed as mean±SEM. Data generated by A. Kimura. C2CR.33 mice were compared to WT littermate control mice by two-way ANOVA with Bonferroni posttests; n=6 per group. * $p<0.05$, ** $p<0.01$.

Although mCPP has a high affinity for the 5-HT_{2C} receptor, it has also been shown to act on other 5-HT receptor subtypes, which can affect behaviour. Hence, a more selective 5-HT_{2C} receptor agonist (RO 60-0175) was administered to determine the extent of 5-HT_{2C} receptor contribution on behavioural responses. This experiment

was undertaken in C2CR.33 mice, backcrossed to C57BL/6 for 6 generations, which under basal conditions do not show any altered behaviour compared to WT mice (see Figure 3.6b). Accordingly results following RO-60 0175 must be interpreted with caution when comparing to those gained from the mCPP injection. RO 60-0175 significantly reduced total locomotion in the OF in both C2CR.33 and WT mice (Figure 3.18; $F_{2,24}$=8.31; p=0.002), however locomotion in both genotypes was equally effected (Figure 3.18; $F_{1,24}$=0.43; p=0.517).

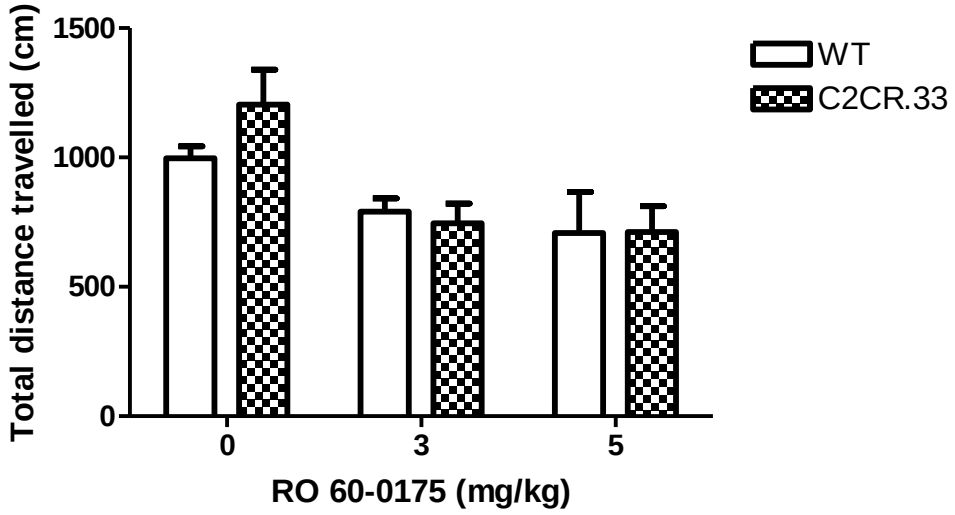


Figure 3.18: Response to RO 60-0175 administration in the open field in male C2CR.33 mice Total locomotion travelled in the OF was reduced following IP injection of RO 60-0175 (3mg/kg or 5mg/kg), in both gentoypes. All mice were males, backcrossed to C57BL/6 for 6 generations. Data are expressed as mean±SEM; C2CR.33 mice were compared to WT littermate control mice by two-way ANOVA with Bonferroni posttests; n=9 per group.

To test constitutive activity of 5-HT_{2C} receptors, an inverse agonist (SB206553) was administered. In the OF, both genotypes of mice became almost completely immobile following SB206553 administration (Figure 3.19, p=0.464). This was unexpected as an inverse agonist should prevent constitutive activity of 5-HT_{2C} receptor and so was predicted to increase locomotion. At this point, further tests were required to determine the reason for this immobility (see Figure 3.22). It should

be noted that within text freezing was classified as maintaining a rigid, fixed position for a period of 10 seconds while a sedation effect would be expected to have the symptoms of drowsiness, so slower movement with short periods of motionlessness.

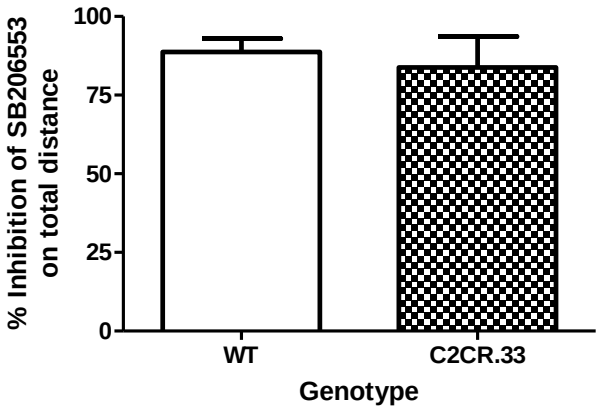


Figure 3.19: Inhibition of distance travelled in the open field following administration of SB 206553 in male C2CR.33

The total distance travelled in the OF was inhibited (% inhibition represents the difference in distance travelled before and after SB206553) in both genotypes in response to the administration of inverse agonist (SB206553). All mice were males, backcrossed to C57BL/6 for 4 generations. Data expressed as mean±SEM; C2CR.33 mice were compared to WT littermate control mice for each region using unpaired Student's t test; n=7 per group.

3.4.2 Response to 5-HT_{2C} receptor ligands in C2CR.10 mice on wheel running behaviour

Effects of 5-HT_{2C} receptor ligands or vehicle on locomotor activity in wheel cages were measured in C2CR.10 mice as they showed a hypolocomotion phenotype under basal conditions. C2CR.10 mice had reduced activity compared to WT mice (Figure 3.20, $F_{1,30}$=5.27; p=0.029). Compared with saline injection, the lower dose of mCPP (0.3mg/kg) had no effect on either C2CR.10 or WT mice. A 3mg/kg dose of mCPP

significantly reduced locomotion in both genotypes (Figure 3.20, effect of drug $F_{2,30}$=3.81; p=0.034) but there was no interaction between drug and genotype ($F_{2,30}$=0.73; p=0.49).

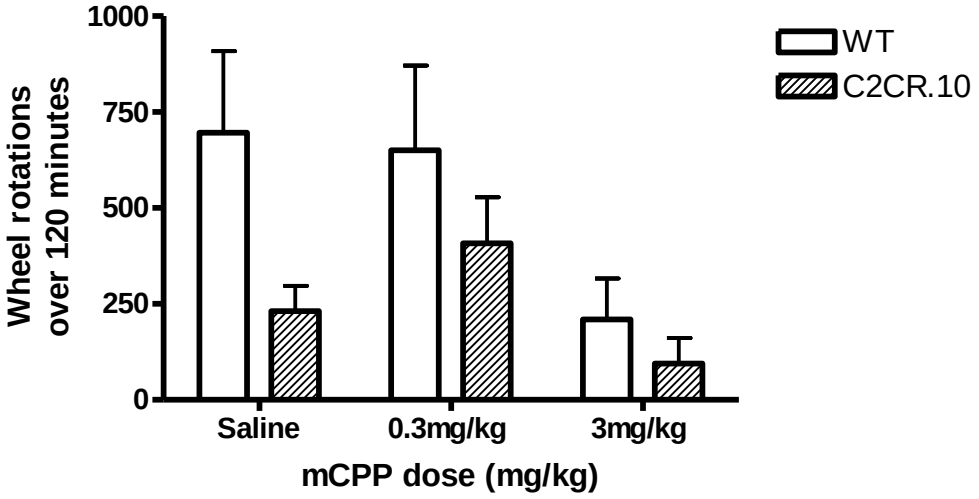


Figure 3.20: Effect of mCPP administration on voluntary wheel running in C2CR.10 mice C2CR.10 mice had reduced locomotion to WT mice following saline administration, 0.3mg/kg mCPP had no effect but 3mg/kg mCPP reduced locomotion in WT mice. All mice were males, backcrossed to C57BL/6 for 3 generations. The effect of mCPP on wheel rotations was measured for two hours following IP injection. Data are expressed as mean±SEM; C2CR.10 mice were compared to WT littermate control mice by two-way ANOVA with Bonferroni posttests; n=6 per group.

In order to further examine whether the reduced wheel running activity was due to higher sensitivity of the receptor to endogenous 5-HT or increased constitutive activity, a selective 5-HT_{2C} receptor antagonist (SB242084) or an inverse agonist (SB206553) were injected 30 (minutes prior to the onset of the dark phase) and activity measured for two hours following dark phase onset. This experiment was planned and carried out in parallel with the administration of SB206553 in C2CR.33

mice; it was not designed to test the unexpected results in Figure 3.19. A dose of the SB242084 previously shown to antagonise 5-HT_{2C} receptors was chosen. Again, locomotion was reduced in C2CR.10 mice compared to WT mice (Figure 3.21; effect of genotype $F_{1,10}$=5.80; p=0.037). However, there was only a trend of effect of drug ($F_{1,10}$=3.64; p=0.085) and there was no interaction between drug administration and genotype ($F_{1,10}$=0.51; p=0.490). This suggests that the hypolocomotion of C2CR.10 mice could be partly to constitutive activity of the overexpressed 5-HT_{2C} receptor rather than increased ligand activation by endogenous 5-HT however the inverse agonist data is required to draw a conclusion.

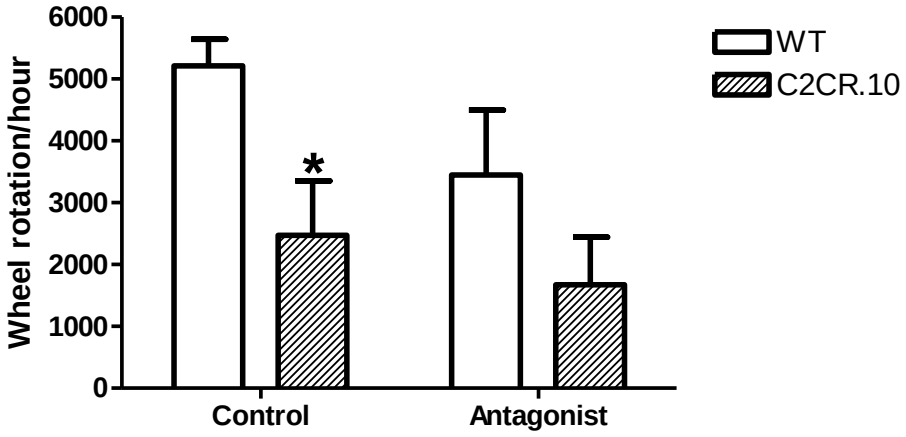


Figure 3.21: Effect of SB242084 administration on voluntary wheel running in C2CR.10 mice
C2CR.10 mice had reduced locomotion to WT mice following vehicle (5%DMSO in water) administration and there was trend for an effect of antagonist administration. All mice were males, backcrossed to C57BL/6 for 3 generations. The effect of SB242084 on wheel rotations was measured for two hours following IP injection. Data are expressed as mean±SEM; C2CR.10 mice were compared to WT littermate control mice by two-way ANOVA with Bonferroni posttests; n=6 per group. (* p<0.05 by unpaired Student's t test)

Administration of the inverse agonist reduced locomotion in both C2CR.10 and WT mice (Figure 3.22a, effect of drug $F_{2,20}$=9.48; p=0.001) similar to the response in C2CR.33 following administration of SB206553. There was an effect of genotype ($F_{1,20}$=5.14; p=0.047) with a dose-dependent effect of SB206553 on locomotion in WT mice (p=0.0013; one-way ANOVA), but not in C2CR.10 mice (p=0.272; one-

way ANOVA). It is possible that the inverse agonist is unable to reduce locomotion in C2CR.10 mice further than the hypolocomotion caused by over-expression of 5-HT_{2C} receptor. These data and the above data (Figure 3.19) suggested that this inverse agonist was acting on receptors other than 5-HT_{2C} receptors. Accordingly, SB206553 was administered to TetO-2CR mice which show negligible expression of the 5-HT_{2C} receptor (see Chapter 5). Locomotion was reduced in both WT and TetO-2CR mice to the same degree (Figure 3.22b; p=0.818. See Chapter 4 for further details) indicating that SB206553 administered intraperitoneally is not specific for 5-HT_{2C} receptors.

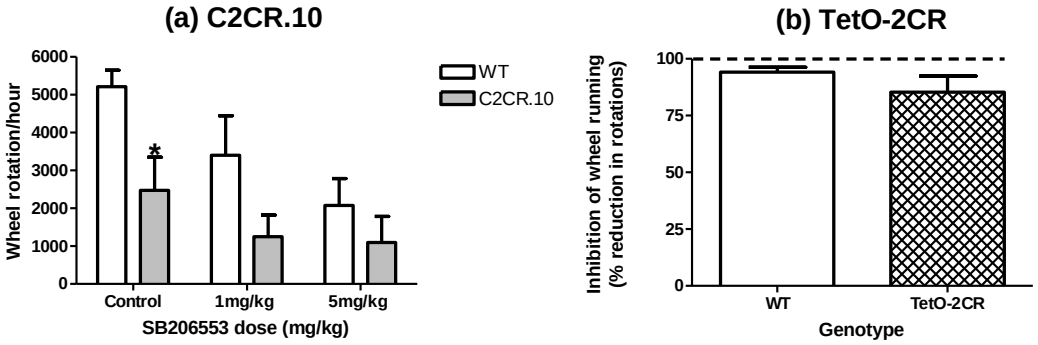


Figure 3.22: Effect of SB206553 administration on wheel running in C2CR.10 or TetO-2CR mice

Compared to vehicle (5%DMSO in water), administration of 1mg/kg or 5mg/kg inverse agonist (SB206553) reduced wheel running (a) in both C2CR.10 and WT mice. Both doses had a similar effect in C2CR.10 mice while there was a dose-dependent effect of SB206553 in WT mice. Male mice backcrossed to C57BL/6 for 4 generations (a). Wheel running was inhibited in both TetO-2CR and WT mice (b) following administration of 5 mg/kg dose of SB206553. The effect of SB206553 on wheel rotations was measured for two hours following IP injection. Data expressed as mean±SEM. C2CR.10 mice were compared to WT littermate control mice by two-way ANOVA with Bonferroni posttests and TetO-2CR mice were compared to WT littermate control mice by unpaired Student's t test; n=6 per group. * $p<0.05$.

3.5 Discussion

3.5.1 5-HT_{2C} receptor mRNA and protein levels in brains of C2CR.10 and C2CR.33 mice during initial characterisation

5-HT_{2C} receptor mRNA expression was significantly increased in both C2CR.33 mice (high over-expressor) and C2CR.10 mice (low over-expressor) limbic regions and the cerebral cortex (C2CR.33 mice only). The transgene generated 5-HT_{2C} receptor mRNA over-expression pattern is more restricted in C2CR.10 mice than in C2CR.33 mice. C2CR.10 mice had over-expression of 5-HT_{2C} receptor in the AcbC, dorsal CA1 field of the hippocampus, dorsal and ventral CA3 field of the hippocampus and dorsal and ventral DG. In addition to these areas, C2CR.33 mice had overexpression in the CPu, AcbSh, primary somatosensory cortex, primary and secondary motor cortex as well as the cortex and BLA. C2CR.33 mice had more regions with overexpression of 5-HT_{2C} receptor mRNA in the hippocampus compared to C2CR.10 mice with elevated levels in the dorsal and ventral CA1, CA2, CA3 fields of the hippocampus and DG. Interestingly, C2CR.10 mice had significantly elevated 5-HT_{2C} receptor mRNA levels in the SN compared to WT mice, but no alterations were found in this region in C2CR.33 mice. This pattern of expression, driven by the calcium-calmodulin-dependent protein kinase IIα promoter (CamKIIα), was similar to the observed pattern of endogenous 5-HT_{2C} receptor expression.

The increased 5-HT_{2C} receptor mRNA levels were translated into increased receptor levels in the forebrain of C2CR.33 mice as demonstrated in the binding study. In C2CR.10 mice, it was not possible to detect an increase in ^{3}H-mesulergine binding in the membrane preparation containing the forebrain, possibly because the preparation also contained the midbrain and the cortex (together constituting a large proportion of the forebrain) and 5-HT_{2C} receptor mRNA levels were unaltered in these regions in C2CR.10 mice. In the hindbrain membrane preparation, 5-HT_{2C} receptor protein did not differ between C2CR.33 and WT mice. However, in C2CR.10 mice a significant increase in binding was found in the hindbrain membrane preparation.

One contributing factor could be the possibility that a larger portion of the raphe nuclei was incorporated into the hindbrain membrane preparation in C2CR.10 preparations compared to C2CR.33 preparations. In the raphe nucleus, 5-HT_{2C} receptor mRNA levels were unaltered in C2CR.33 mice but it is possible they are elevated in C2CR.10 mice, which was not examined in this line due to a lack of mice. A known problem in transgenic models generated using the CamKIIα promoter (which is expected to cause alterations in the forebrain) is the expression of the transgene in the cerebellum and this is may be the case in C2CR.10 mice. Unfortunately due to a shortage of animals, analysis of the cerebellum to assess 5-HT_{2C} receptor mRNA levels could not be undertaken in C2CR.10 mice.

3.5.2 Behavioural phenotype in 5-HT_{2C} receptor overexpressing C2CR.10 and C2CR.33 mice during initial characterisation

Due to the regions of 5-HT_{2C} receptor over-expression in both C2CR.10 and C2CR.33 mice in the limbic system and cortex, an increase in anxiety-like behaviour, depressive like behaviour and hypolocomotion were anticipated.

During the initial characterisation a slight increase in anxiety-like behaviour in response to novel environments containing a component of stress, was found in both C2CR.33 and C2CR.10 mice. This consistent phenotype seen in both lines suggests this was a result of the 5-HT_{2C} receptor over-expression rather than disruption of an unknown gene by the random chromosomal insertion of the transgene. The behavioural phenotype of C2CR.33 mice was greater than that of C2CR.10 mice with greater anxiety-like behaviour than C2CR.10 mice. Hypolocomotion in response to a novel environment was observed in C2CR.33 mice only.

The increase in anxiety-like behaviour in the absence of exogenous ligand suggests that receptors in C2CR.10 and C2CR.33 mice may be already activated by low levels of endogenous ligand or may be constitutively active. Either (or both) could result from an altered ratio of unedited to edited 5-HT_{2C} receptor isoforms in these lines as the transgene generated receptors are not capable of being edited. Alternatively, as the 5-HT_{2C} receptor influences the HPA axis through the regulation of CRF in hypothalamic regions (Heisler *et al*., 2007a), and increased anxiety-like behaviour may be caused by altered HPA axis activity. However, as 5-HT_{2C} receptor mRNA levels were unaltered in hypothalamic regions and it had previously been shown that under both basal and stressed conditions corticosterone levels were similar in both C2CR.10 and C2CR.33 mice compared to WT mice (Kimura *et al*., 2009), therefore it is unlikely that the anxiety phenotype results from a hypersensitive HPA axis.

To date, the literature supports the finding that increased 5-HT_{2C} receptor signalling elevates anxiety-like behaviour, while antagonists act as anxiolytics (Kennett *et al*., 1989, Jenck *et al*., 1998, Harada *et al*., 2008). 5-HT_{2C} receptor knock-out (KO) mice had an anxiolytic phenotype (Heisler *et al*., 2007b) which concurs with the literature on the effect of antagonists.

Hypolocomotion resulted from forebrain 5-HT_{2C} receptor over-expression, although hypolocomotion was dependent on the line of C2CR mice and the behavioural test used. Over-expression of 5-HT_{2C} receptors in C2CR.33 mice caused hypolocomotion in the novel environment of the OF. However, this was not replicated in C2CR.10 mice (Kimura *et al*., 2009). This could be explained by the elevation in 5-HT_{2C} receptor mRNA levels in the cortex (responsible for locomotor functions) only being present in C2CR.33 mice and not in C2CR.10 mice.

Voluntary wheel running allows locomotion to be examined without the stress of a novel environment although it is dependent on the mice voluntarily exercising. No difference in total wheel running was found in the C2CR.33 mice (A. Kimura, unpublished data) compared to WT mice. In contrast, a strong hypolocomotor phenotype was found in C2CR.10 mice in this test. One possible explanation for this

difference between the lines is that 5-HT_{2C} receptor protein levels were elevated in the hindbrain membrane preparation of C2CR.10 mice, but not C2CR.33 mice. Dopamine (DA) is involved in locomotion and the 5-HT_{2C} receptor influences DA release. It is believed that an inhibitory control on both the mesolimbic and nigrostriatal DA systems by 5-HT occurs through the 5-HT_{2C} receptor (Di Matteo *et al*., 2001; Di Matteo *et* al., 2002; De Deurwaerdere *et al*., 2004; Higgins and Fletcher., 2003; McMahon *et al*., 2001). It is well established that DA depletion by a gradual degeneration of SN compacta (SNc) cells projecting to the STR results in loss of movement in Parkinson's disease (Bossy-Wetzel *et al*., 2004). Functioning of the substantia nigra (SN) is vital as it is one of three regions in the brain containing primary DA synthesising cells. 5-HT_{2C} receptor mRNA levels were elevated in the SN only in C2CR.10 mice, so it is possible that the increase in 5-HT_{2C} receptors in the SN may influence DA levels. The relationship between 5-HT_{2C} receptor activation in the SN, leading to inhibition of DA release in the nucleus accumbens and prefrontal cortex by reduced firing rate and bursting-activity of DA-containing neurons in the ventral tegmental area (VTA) could be the mechanism by which this hypoclocomotion is achieved and this will be discussed in greater detail in chapter 5, where aspects of the DA system are examined.

The different behavioural tests used allow different parts of the CNS to be tested. Therefore, the increase in 5-HT_{2C} receptor in the SN in C2CR.10 mice (but not C2CR.33 mice) may underlie the hypolocomotor phenotype in voluntary wheel running cages and the increase in 5-HT_{2C} receptor in the cortex of C2CR.33 (but not C2CR.10) may be the basis for the difference in locomotion in the novel environment.

3.5.3 The effect of strain background on the phenotype of 5-HT_{2C} receptor over-expressing mice

In order to establish a good model for carrying out behavioural tests it is vital that the genetic background of transgenic mice is the same as their WT controls as different strains have markedly different behavioural traits. Thus behavioural traits may be due to mixed genetic backgrounds in transgenic mice rather than the transgene itself, or could reflect the interaction of different backgrounds with the transgene. Backcrossing onto the C57BL/6 line was chosen, as this line has been well characterised in behavioural tests and is known to breed consistently well. Mouse strains which show a high level of physical activity are a good choice for examining a putative hypolocomotor phentoype and/or increased anxiety-like behaviour, both of which were hypothesised to result from 5-HT_{2C} receptor over-expression in this model. C57BL/6 mice show high levels of locomotion (ambulation) in the OF and low levels of anxiety-related measures. Following eight generations of backcrossing onto C57BL/6 the line is considered to be congenic with C57BL/6 genetic background which, as mentioned above, is desirable for behavioural testing so any alterations in phenotype found likely due to the transgene instead of a mixed genetic background.

During the backcrossing procedure, C2CR mice were retested to confirm the behavioural phenotype found in the initial characterisation. However, both the anxiety-like phenotype and hypolocomotion of C2CR.33 mice were no longer detectable following backcrossing and similarly, the strong hypolocomotion phenotype of C2CR.10 mice in wheel running cages disappeared (due to a shortage of animals it was not possible to retest this line in the mazes). It remains possible that although the previous phenotype was no longer detectable, other similar tests may be able to detect a behavioural difference in these lines of mice. However, it is also possible that the phenotype may be due to a mixed background. The possibility of transgene silencing was ruled out.

The alteration in genetic background must be considered as a major factor in the loss of phenotype. Early generations in the backcrossing process will have a mixed behavioural phenotype due to the lines used in the production of this model; CBA, C3H and C57BL/6. All of these lines have similar characteristics in behavioural tests (for example percentage time in open quadrants of elevated zero maze; 12.7% in C57BL/6, 23.6% in CBA and 28.4% in C3H, from Mouse Phenome Database, Jax Laboratories). In both the novel environment with a component of stress (OF) and in voluntary wheel running, the loss of locomotor phenotype is being generated by a reduction in total locomotion in the WT mice. It is unlikely that this reduction is due to the increased genetic background of C57BL/6 as a similar reduction would be anticipated in C2CR.10 and C2CR.33 mice. All of the mouse lines used in generating C2CR mice have similar locomotor activity, for example horizontal movement is around 2513 in C3H, 2889 in CBA and 3226 in C57BL/6 (from Mouse Phenome Database, Jax Laboratories) so if anything, an increase in general locomotion would have been anticipated with the increased influence from C57BL/6.

Additional tests were carried out on the later generations of C2CR.33 mice in case another aspect of mood was altered, such as fear responses. 5-HT release and activation of 5-HT_2 receptors, particularly within the amygdaloid complex, have been associated with anxiogenic behaviours and emotional stress (Chaouloff *et al*., 2000; Kawahara *et al*., 1993; Stein *et al*., 2000) as well as facilitating conditioned fear in rodents (Graeff *et* al., 1997; Mora *et al*., 1997) and learned fear in humans (Graeff *et al*., 1996). The 5-HT_{2C} receptor has been shown to have an anxiolytic effect in conditioned anxiety (Davis *et al*., 1986) and activation within the basolateral amygdala (BLA) potentiates innate fear responses (Campbell and Merchant., 2003). Because C2CR.33 mice have increased 5-HT_{2C} receptor mRNA levels in the amygdaloid complex, it was hypothesised that conditioned fear responses may be increased. This was not found to be the case. The regulation of fear responses is complex and 5-HT_{2C} receptor activation has region-specific consequences, with inhibition of innate fear responses in the dorsal periaqueductal gray region but potentiation of conditioned fear in the amygdala (Graeff *et al*., 1997).

Another point of interest is that the 5-HT_{2C} receptor is expressed on both the projection neurones and interneurons in the BLA (Stein *et al*., 2000) which could have opposing actions on GABA release (Rainnie *et al*., 1999; Stutzmann *et al*., 1999). Due to the complex regulation of fear responses and dependency on the specific type of anxiety, for example learned or innate fear, further tests would have to be carried out to exclude an effect of forebrain over-expression of 5-HT_{2C} receptors upon fear responses.

The literature on the effects of 5-HT_{2C} receptor agonists and antagonists upon depressive-like behaviour is conflicting. Antagonists, such as the potent 5-HT_{2C} receptor antagonist, S32006 (Dekeyne *et al*., 2008) in addition to being anxiolytic, have antidepressive properties (Millan *et al*., 2006). DBA/2N mice have lower brain 5-HT levels and are found to be insensitive to citalopram, a SSRI, but when a 5-HT_{2C} receptor antagonist is administered in combination with citalopram this restores its antidepressant effect (Calcagno et al, 2008). Agomelatine, a 5-HT_{2C} receptor antagonist and melatonin receptor agonist, is effective in the treatment of major depressive disorder as well as SAD disorder (Millan *et al*., 2005; Pjerk et al 2006), and has been found to improve sleep disturbances (Pandi-Perumal *et al*., 2009). There is evidence that agonists of the 5-HT_{2C} receptor may also be efficacious antidepressants (Dunlop *et al*., 2006). In C2CR.33 mice, over-expression of 5-HT_{2C} receptor in the forebrain did not alter depressive-like behaviour. This result is not conclusive as only one test of depressive-like behaviour was used. Some 5-HT_{2C} receptor antagonists are inactive in tests of antidepressant, anti OCD and antipanic activity (Jenck et al 1998) indicating that the effects may be due to off-target effects of the antagonist or that 5-HT_{2C} receptor involvement is not always picked up in behavioural testing. Hence further tests are required to confirm this absence of depressive-like behaviour in this model.

The general ability and balance of the mice was tested using the rotorod in order to exclude this as a cause for the hypolocomotion previously found. C2CR.33 mice performed just as well as WT mice in this test and no obvious differences in activity in home cages were noticed in C2CR.10 or C2CR.33 mice (personal observation).

This suggests that the hypolocomotion found during the intitial characterisation was due to mixed genetic background.

There are many possible reasons for the loss of phenotype following backcrossing, the genetic background of the mice has already been discussed. The most easily tested is that the transgene may be silenced by mutations or epigenetic silencing through generations. This can be ruled out in C2R.33 mice as the generation 6 C2CR.33 mice still had increased 5-HT_{2C} receptor mRNA in the same regions as found during the initial characterisation as well as increased 5-HT receptor levels in the forebrain.

One major factor to consider is that there was a move in animal facilities at the time when the phenotype was lost. The new facility is much bigger, busier and noisier than the previous one, possibly stressful for the mice, with consequent effects on behaviour. To assess whether the business of the holding rooms was causing the loss of phenotype, following weaning animals were moved to a secluded quiet room where access was restricted. This did not restore the phenotype, even after several months. In addition, breeding of the mice in the quiet area of the unit (to eliminate any possibility of stress caused by moving animals within the unit) had no impact on the behavioural phenotype.

The area to consider in this alteration in WT locomotion is the HPA axis which is influenced by the 5-HT_{2C} receptor as previously mentioned. The decreased locomotion in the OF in WT mice over generations could be due to alterations in the HPA axis resulting from backcrossing onto the C57BL/6 background. However, in all lines involved in the generation of this model (C57BL/6, CBA and C3H) adrenal weights and corticosterone secretion are similar and therefore altering the genetic background of the mice by backcrossing onto C57BL/6 would not be expected to alter either adrenal weights or corticosterone secretion. Effects of transgenes can greatly depend on the genetic background of the strain with some non sense mutations being lethal on one strain but not on another (for example, 11βHSD2 KO mouse). In the OF the total locomotion in C2CR.33 mice remained constant across

generations while WT mice locomotion altered. It would be expected that a similar alteration in locomotion would be found in both C2CR.33 and WT mice would be expected if the change was due to backcrossing onto the C57BL/6 background. It is possible that the transgene is maintaining locomotion regardless of the genetic background across generations in C2CR.33 mice. Voluntary wheel running has been shown to have an antidepressant effect and alters both adrenal weight and HPA axis responses initially and it is possible that these potential alterations in could affect wheel running behaviour in C2CR.10 and WT mice.

There may have been compensation for the increase in 5-HT_{2C} receptor within the serotonin system which occurred over the generations during backcrossing. For example, a reduction or increase in another 5-HT receptor could normalise the behavioural phenotype. This will be discussed in Chapter 5, where both the 5-HT and DA systems were examined.

3.5.4 Response to 5-HT_{2C} receptor agonist and antagonist administration in C2CR.10 and C2CR.33

The injection procedure for ligand administration is a stressful one in for mice and vehicle injection caused hypoactivity in C2CR.10, C2CR.33 and WT mice, in both the OF and voluntary wheel running tests. The stress of removing mice from their wheel cages and the injection inhibited their previously consistent activity patterns. The extent of this hypoactivity varied and, in some cases, was so severe that no further inhibition of activity could be observed following agonist administration. The acute nature of the effect (under thirty minutes) excluded a genomic action of, for example, corticosterone (which would most probably take over forty minutes). The hypolocomotion response plausibly results from a direct effect of 5-HT release, or other neurotransmitter pathways, rather than the transgene, as C2CR.10. C2CR.33 and WT mice were equally affected.

3.5.4.1 Response to 5-HT_{2C} receptor agonists in C2CR.33 mice in a novel environment

The mixed 5-HT_{2C} receptor agonist, mCPP, administered at levels previously shown to reduce locomotion in mice, reduced activity in both voluntary wheel running and in a novel environment (OF). In the novel environment, although effective in reducing locomotion in WT mice, mCPP was ineffective in C2CR.33 mice. This suggests that mCPP is unable to further decrease locomotion below that seen with vehicle in this line. In 5-HT_{2C} receptor KO mice, mCPP actually elicits hyperactivity (Heisler & Tecott., 2000; Dalton *et al*., 2004) which could be due to activation of 5-HT_{1B} and 5-HT_{2A} receptors which is unmasked in the absence of 5-HT_{2C} receptors. Hence the more specific 5-HT_{2C} receptor agonist, RO 60-0175, was used in the next experiment. A sedative-like effect of RO 60-0175, with no effect on anxiety, has been reported (Kennett et al 2000). RO 60-0175 administration reduced locomotion of WT and C2CR.33 mice. It is possible that a sedative like effect could be the reason for the reduced locomotion of C2CR.33 mice but in order to determine this, a dose-response should be carried out. It is possible that hypolocomotion is not a sensitive measure in this test and so an antagonist should be administered to block the effect of the agonist. The administration of 5-HT_{2C} receptor agonists reduced locomotion in both WT and transgenic mice to some extent, therefore there is no evidence in these experiments for altered sensitivity of 5-HT_{2C} receptor.

3.5.4.2 Response to 5-HT_{2C} receptor ligands in C2CR.10 mice in voluntary wheel running cages

At the higher dose, the mixed 5-HT receptor agonist, mCPP, reduced wheel-running activity equally in both C2CR.10 and WT mice although interestingly the level of agonist inhibition of activity did not differ between the genotypes regardless of the different receptor expression levels. The antagonist (SB242084) failed to elevate activity levels in both C2CR.10 and WT mice at a dose previously shown to be

inhibitory *in vivo* (Kennett *et al*., 1997; Nonogaki *et al*., 2007). This suggests that the hypoactivity of C2CR.10 mice could be due to constitutive 5-HT_{2C} receptor activity and not to ligand activation by the low levels of endogenous 5-HT present. In order to assess the constitutive activity element an inverse agonist can be used which antagonises both the ligand-activated receptor activity in addition to reversing constitutive activity. The inverse agonist SB206553 had previously been used *in vivo* successfully in rat, human and frog (Berg et al 1999; Chanrion et al 2007). Surprisingly, instead of restoring activity levels, administration of SB206553 virtually abolished all movement in wheels or a novel environment, regardless of genotype. In rats the standard dose of SB206553 used is 5mg/kg, due to the non-specific effect found by the 5mg/kg dose in these mice it was possible that (considering the bell-shaped dose response curve) too high a dose had been given. However, a lower dose of SB206553 (1mg/kg) also reduced locomotion. Notably, a similar effect was found in 5-HT_{2C} receptor-deficient mice (TetO-2CR mice; Chapter 4) suggesting that in mice, this effect of SB206553 is independent of 5-HT_{2C} receptor. This inverse agonist also binds to 5-HT_{2B} receptor which in the brain has similar effects to 5-HT_{2C} receptor. However, the 5-HT_{2B} receptor is also expressed in the periphery where it is found is on motor neurones. It could therefore affect movement through a peripheral action due to administration intraperitoneally. It might also be acting on 5-HT_{2A} receptors in the mouse brain due to large homologous regions with the 5-HT_{2C} receptor, predicted to reduce movement.

Because SB206553 is not a specific 5-HT_{2C} receptor inverse agonist in mice, this experiment was unable to determine if the hypolocomotion is due to increased constitutive activity or enhanced sensitivity to endogenous ligand. It is likely that a combination of constitutive activity and enhanced endogenous ligand sensitivity will be contributing to the phenotype.

3.5.7 Concluding Remarks

The transgene resulted in 5-HT_{2C} over-expression in a restricted region-specific manner in C2CR.10 compared to C2CR.33 mice which had over-expression in most brain regions examined. C2CR.33 mice had higher over-expression of 5-HT_{2C} receptor compared to C2CR.10 mice. 5-HT_{2C} receptor binding sites were increased in the forebrain of C2CR.33 mice and in the hindbrain of C2CR.10 mice. Both C2CR.10 and C2CR.33 lines of mice showed anxiety-like behaviour and hypolocomotion; an effect that was lost on subsequent backcrossing to C57BL/6 and there was no indication of altered depressive-like or fear behaviour. This loss of behavioural phenotype was not due to transgene turn off as 5-HT_{2C} receptor mRNA and binding sites were still elevated in C2CR.33 mice following backcrossing to C57BL/6 for 7 or 6 generations. The reason for the loss of phenotype is therefore unclear, although genetic background or environment may be a factor.

CHAPTER 4:

CHARACTERISATION OF A MOUSE DEFICIENT IN THE 5-HT_{2C} RECEPTOR THROUGHOUT THE BRAIN

Aims

This experimental chapter examines the 5-HT_{2C} receptor under-expressing mouse line and has the following aims:

1. To determine the extent of the deficiency in 5-HT_{2C} receptors in the TetO-2CR mouse line
2. To establish any behavioural phenotype that may be present as a result of the 5-HT_{2C} receptor deficiency and examine functionality of the 5-HT_{2C} receptor using ligand studies
3. To consider if the deficiency in the 5-HT_{2C} receptor could alter the regulation of either the food entrainable oscillator or the light entrainable oscillator

It was hypothesised that 5-HT_{2C} receptor would be decreased causing hyperlocomotion, hyperphagia and reduced anxiety-like behaviour.

4.1 Introduction

5-HT_{2C} receptors have been implicated in many behaviours including the regulation of mood, food intake and locomotion (Gleason *et al*., 2001; Schuhler *et al*., 2005; Harada *et al*., 2008). Mice that lack 5-HT_{2C} receptors (5-HT_{2C} receptor KO mice) are hyperphagic, developing obesity in later life (Tecott *et al*., 1995), have an anxiolytic phenotype (Heisler *et al*., 2007b), show hyperlocomotion (Heisler & Tecott., 2000; Dalton *et al*., 2004) and obsessive compulsive behaviour (Chou-Green *et* al., 2003). In many cases creating a knockout model of a particular receptor provides an indispensible tool to elucidate its pathophysiological function. This study uses mice, termed TetO-2CR mice (developed by McColl, Chapman, Homes and Melton, see Chapter 2), in which the 5-HT_{2C} receptor has been targeted using a construct containing a tetracycline activator (TetA) hence nomenclature of TetO-2CR but in this case being used as an under-expressing model. In TetO-2CR mice, the expression of the 5-HT_{2C} receptor is driven by the normal 5-HT_{2C} receptor promoter and, unexpectedly, a deficiency in the 5-HT_{2C} receptor was produced throughout the brain. This model has the advantage of having low levels of the receptor throughout development as opposed to no 5-HT_{2C} receptors which can be a potential problem in knockout models (KO). This is the case in the 5-HT_{2C} receptor KO mice as a percentage of these animals suffer from spontaneous seizures and death (Tecott *et al*., 1995). TetO-2CR mice are novel as they are resistant from spontaneous seizures. The first aim of this chapter was to establish the extent of this deficiency and the effect this has on behaviour. Secondly, the loss of receptor expression may result in compensatory alterations in other systems and this will be considered. The final part of this chapter considers whether a deficiency in 5-HT_{2C} receptor could alter the regulation of the light entrainable oscillator (LEO) and/or the food entrainable oscillator (FEO).

4.2 Basic Analysis of model

The construct used to create the TetO-2CR mice was targeted to the 5-HT_{2C} receptor gene. The targeting cassette was found to be passed through the generations with a ratio of 2 WT: 1 TetO-2CR mice per litter, with the average litter size being 9 pups. Basic analysis of model had been carried out by A. Kimura which showed that until 23 weeks of age TetO-2CR mice had a similar BW growth curve to WT mice (Figure 4.1a) after which BW diverged and TetO-2CR mice became significantly heavier than WT mice (Figure 4.1b; p=0.005). TetO-2CR males were also shown to have a greater food intake (Figure 4.1c; p=0.003).

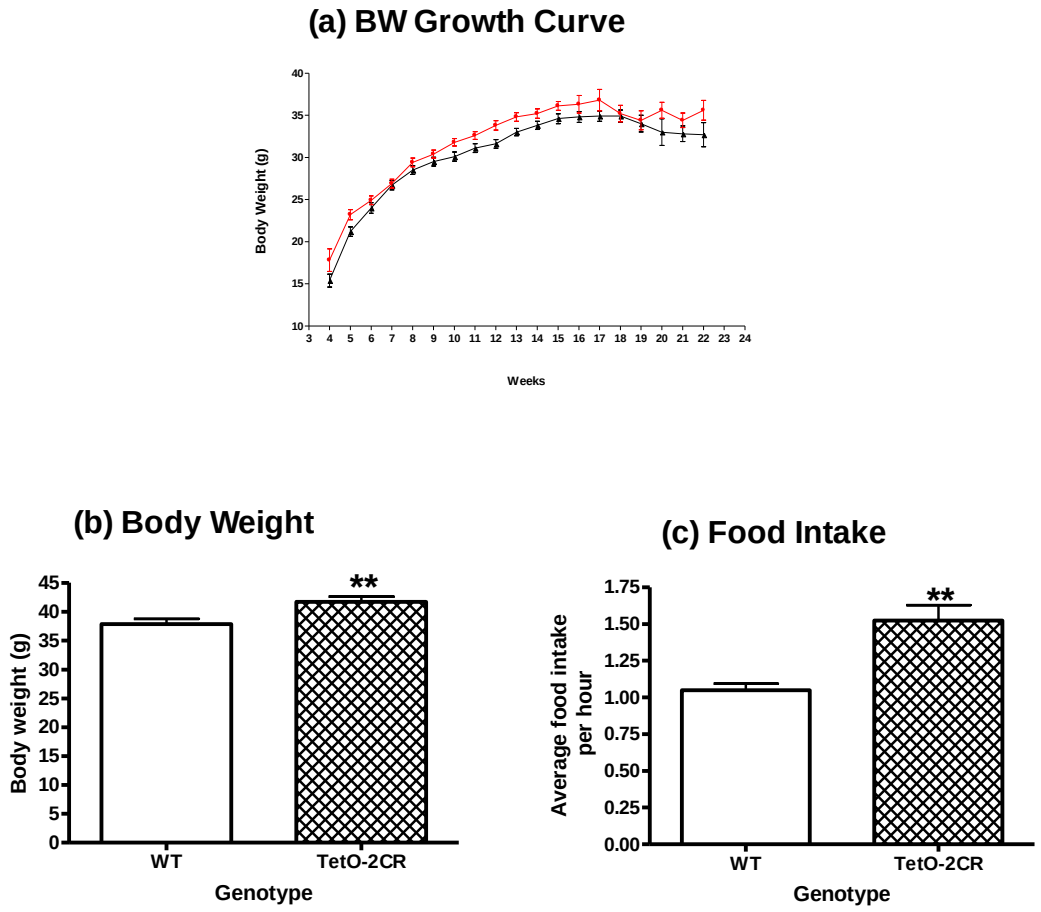


Figure 4.1: Body weight and food intake in TetO-2CR males
Effect of genotype on (a) Body weight growth curve (WT = black; TetO-2CR = red), (b) body weight at 24 weeks and (c) average hourly food intake in 24 week old TetO-2CR males, generation 2 of backcrossing onto C57BL/6. Data are expressed as mean±SEM and TetO-2CR males are compared to WT littermate controls by unpaired Student's T-test; n=6-19 per group. ** p <0.01.

4.2.1: Tetracycline activator (tTA) mRNA is present in TetO-2CR mice

In the TetO-2CR mice the tetracycline activator (tTA) protein is encoded by the targeting construct and transcription of tTA protein is driven by the 5-HT_{2C} receptor promoter. The principle behind this model is that the tTA protein produced binds to the tetracycline (Tet) operon and amplifies promotion of 5-HT_{2C} receptor production. In TetO-2CR mice although tTA is expressed, unexpectedly the result of this targeting was reduced expression of the 5-HT_{2C} receptor. The presence of tTA mRNA can, however, be used to indicate the regions where alteration in the 5-HT_{2C} receptor levels would be anticipated due to the fact that tTA protein is not normally present in mouse brain. tTA mRNA was found in TetO-2CR mice with only nonspecific levels being found in WT mice in regions where the 5-HT_{2C} receptor is expressed (Figure 4.2; $F_{1,30}$=545.96; p<0.0001) and there was an interaction between genotype and region (Figure 4.2; $F_{4,30}$=6.55; p=0.0007).

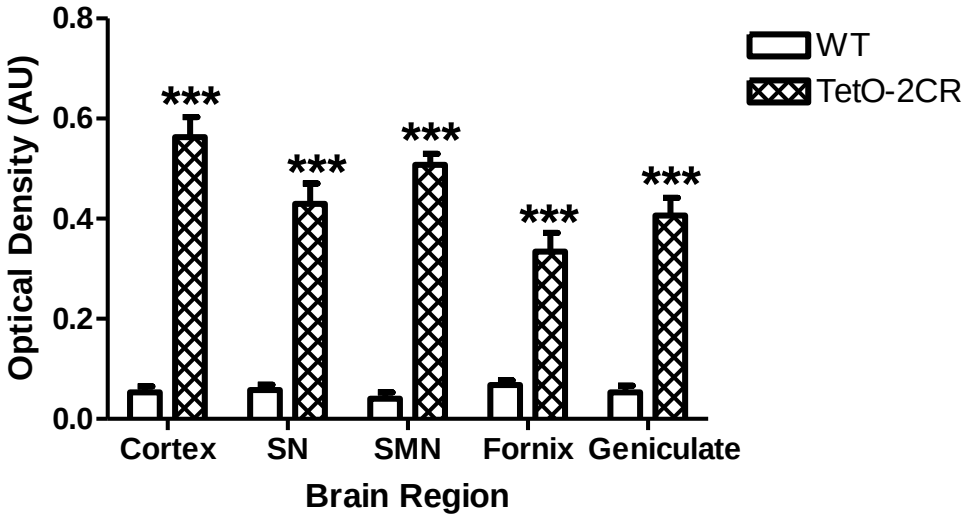


Figure 4.2: Tetracycline activator (tTA) mRNA expression in adult male TetO-2CR mice tTA mRNA was present in TetO-2CR mice indicating the presence of the targeting cassette while only a nonspecific signal was found in WT mice. Expression levels were measured by optical densitometry of *in situ* hybridisation autoradiographs. Data are expressed as mean±SEM in males backcrossed to C57BL/6 for 3 generations. TetO-2CR mice compared to WT littermate control mice by two-way ANOVA with Bonferroni posttests; n=6 per group. SN = Substantia Nigra; SMN = Supramammillary nucleus. *** p<0.001.

4.2.2 TetO-2CR mice show reduced expression of 5-HT_{2C} receptor mRNA throughout the brain

In situ hybridisation was used to establish whether 5-HT_{2C} receptor mRNA expression was altered as a result of the gene targeting in TetO-2CR mice. This technique allows gene quantification of 5-HT_{2C} receptor mRNA levels in brain sections and clearly shows the expression pattern within these sections. TetO-2CR mice had significantly reduced 5-HT_{2C} receptor mRNA levels compared to WT mice in all regions of the brain examined with the exception of the primary somatosensory cortex, primary and secondary motor cortex and the CA1 field of the dorsal hippocampus where the decrease did not reach significance probably because levels of 5-HT_{2C} receptor mRNA expression in these regions is low in WT mice (Figure 4.3). Firstly, in regions of the frontal forebrain such as the caudate putamen (CPu) and nucleus accumbens core (AcbC) and shell (AcbSh) TetO-2CR mice were found to have significantly reduced 5-HT_{2C} receptor mRNA levels with a significant interaction of genotype and region (Figure 4.3a; $F_{4,25}$=8.2; p=0.0002). In more posterior forebrain regions such as the dorsal hippocampus (CA1), amygdala and choroid plexus (CP) 5-HT_{2C} receptor mRNA levels were reduced in TetO-2CR mice with a significant effect of the genotype of the mouse and its interaction with the regions tested (Figure 4.3b; $F_{5,36}$=6.56; p=0.0002). Finally in midbrain regions such as the substantia nigra (SN) although no significant interaction of genotype and region was found (Figure 4.3c; $F_{4,35}$=2.44; p=0.0653) there was a significant effect of genotype (Figure 4.3c; $F_{1,35}$=98.62; p<0.0001).

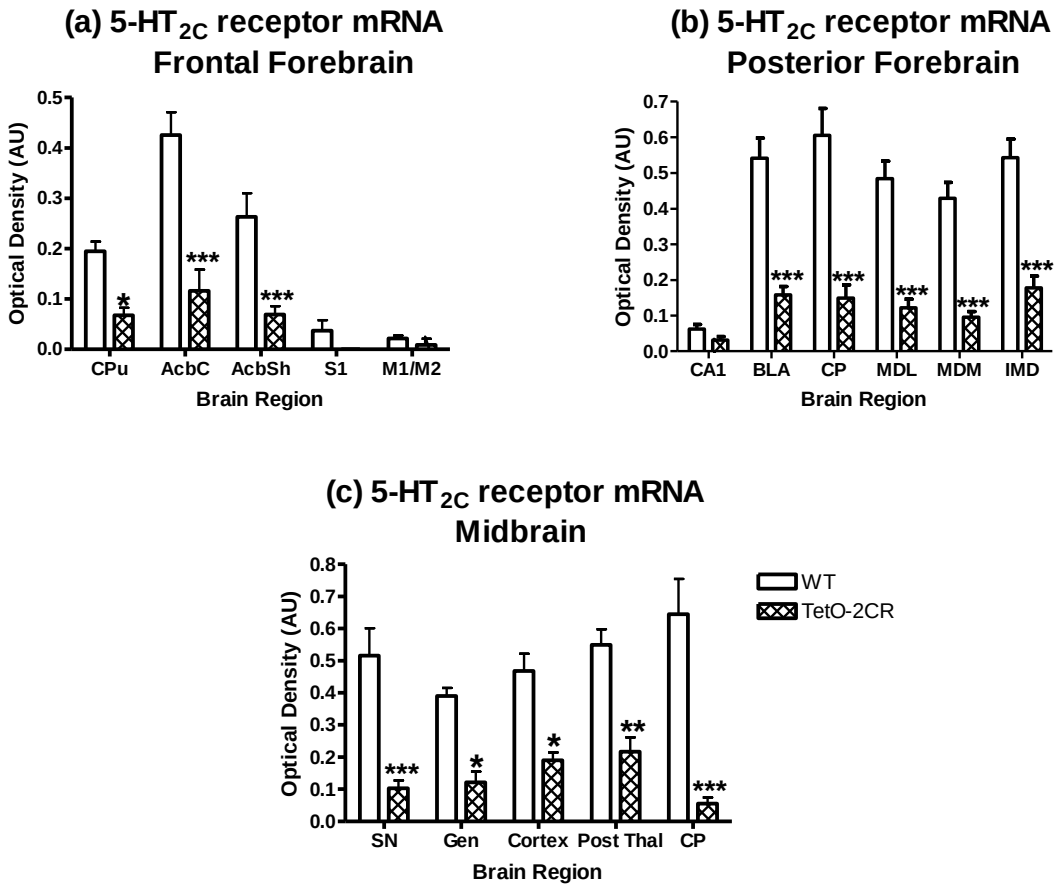


Figure 4.3: 5-HT$_{2C}$ receptor mRNA expression frontal to posterior in adult male TetO-2CR mice

5-HT$_{2C}$ receptor mRNA expression is reduced in TetO-2CR mice compared to WT littermate controls in (a) frontal forebrain, (b) posterior forebrain and (c) midbrain. Expression levels were measured by optical densitometry of *in situ* hybridisation autoradiographs. Data are expressed as mean±SEM in males backcrossed to C57BL/6 for 2 generations and analysed by unpaired, 2-way ANOVA with Bonferroni posttests; n=5 per group. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

CPu = caudate putamen; AcbN = nucleus accumbens core; AcbSh = nucleus accumbens shell; S1= primary somatosensory cortex; M1/M2 = primary/secondary motor cortex; CA1 = CA1 field of the dorsal hippocampus; BLA = basolateral amygdaloid nucleus (anterior); MDL = mediodorsal thalamic nuclei (lateral); MDM = mediodorsal thalamic nuclei (med); IMD = intermediodorsal thalamic nuclei; SN = Substantia nigra; Gen = geniculate; Post Thal = posterior thalamus; CP = choroid plexus.

4.2.3: 5-HT_{2C} receptor binding sites are decreased in TetO-2CR mice

It is vital to establish if the decrease in 5-HT_{2C} receptor mRNA in TetO-2CR mice found by *in situ* hybridisation (section 4.2.2) is translated to the protein level. In order to assess this, 5-HT_{2C} receptor number was measured with a [^{3}H]-mesulergine binding assay in membrane preparations from the midbrain. Consistent with reduced 5-HT_{2C} receptor mRNA level in Teto-2CR mice, [^{3}H]-mesulergine binding was found to be significantly reduced (80%) in TetO-2CR mice compared to WT mice (Figure 4.4; p=0.013).

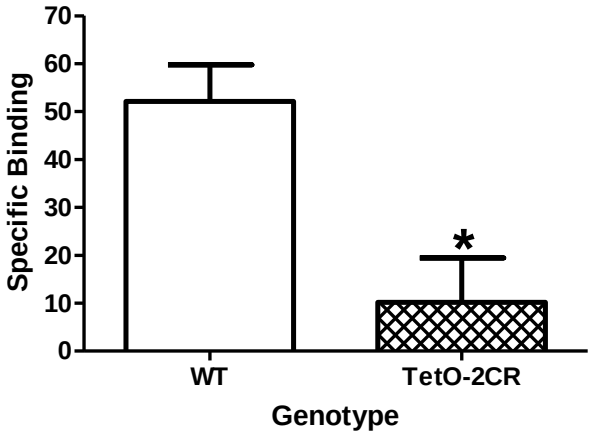


Figure 4.4: 5-HT_{2C} receptor number in the midbrain of adult male TetO-2CR mice 5-HT_{2C} receptor number was reduced in TetO-2CR mice compared to WT mice, determined using [^{3}H]-mesulergine binding in membrane preparation from midbrain homogenates. Data are expressed as mean±SEM in males backcrossed to C57BL/6 for 5 generations and analysed by unpaired Student's t test; n=3 per group. * p<0.05

4.2.4: Body Weight and Food Intake are increased in TetO-2CR mice

The 5-HT_{2C} receptor is involved in the regulation of food intake (FI) with 5-HT_{2C} receptor agonists being appetite suppressants (Schuhler *et al*., 2005). TetO-2CR mice had significantly increased food intake compared to WT mice (Figure 4.5a; p=0.0001 average intake/day corrected for BW measured over 7 days) and were heavier than WT mice by 7 months of age (Figure 4.5b; p=0.006).

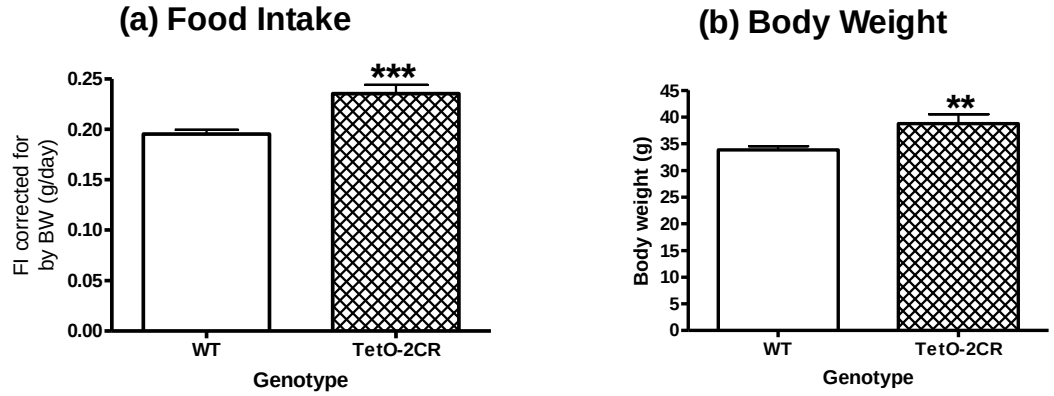


Figure 4.5: Food intake and body weight in male TetO-2CR mice
TetO-2CR 7 month old mice have increased average food intake and body weight compared to WT littermate control mice following backcrossing to C57BL/6 for 3 generations. Data are expressed as mean±SEM and analysed using an unpaired Student's t test; n=14-24 per group. ** p<0.01; *** p<0.001.

4.2.5: 5-HT_{2C} receptor deficiency in TetO-2CR mice causes no alteration in anxiety-like behaviour or depressive-like behaviour in this study

The 5-HT_{2C} receptor has been implicated in the control of mood and anxiety, with agonists reported to be anxiogenic while antagonists are anxiolytic (see Chapter 1). Accordingly the elevated plus maze (EPM) and the open field (OF) tests were used to examine if anxiety-like behaviour was altered in TetO-2CR mice. These are well

established tests of anxiety-like behaviour in mice and test the natural instinct of rodents to avoid brightly light exposed regions of the mazes. In the EPM increased anxiety-like behaviour is indicated by a decrease in exploration, time and/or distance travelled in the elevated and exposed light open arms. The OF works on a similar principle with decreased time and/or distance travelled in the exposed, brightly lit central region of the maze indicating increased anxiety-like behaviour. Both of these measures, percentage time and percentage distance travelled, must be considered before a conclusion can be drawn for both the EPM and the OF. In the EPM, both genotypes spent a similar percentage of time (Figure 4.6a; p=0.612) and travelled a similar distance (Figure 4.6b; 0.525) in the open arms and travelled a similar total distance around the maze (Figure 4.6c; p=0.659). The behaviour of mice in the maze (stretch attend and rearing) was similar between TetO-2CR and WT mice (Figure 4.6d; effect of genotype $F_{1,60}$=2.48; p=0.121), indicating that there is no alteration in anxiety-like behaviour in this test. Unexpectedly TetO-2CR mice showed a trend toward spending less time in the inner zone of the OF compared to WT mice (Figure 4.6e; p=0.0995). However, both TetO-2CR and WT mice travelled a similar distance in the inner zone (Figure 4.6f; p=0.541), although TetO-2CR mice have a trend to reduced total distance travelled around the maze compared to WT mice (Figure 4.6g; p=0.073). During the OF test, a significant interaction of the genotype and behaviour was found (Figure 4.6h; interaction of genotype and behaviour $F_{2,48}$=6.46; p=0.003) with TetO-2CR mice showing significantly less rearing which could indicate reduced exploratory or escape behaviour, but similar grooming levels compared to WT mice. Taken together, the reduction in 5-HT_{2C} receptor throughout the brain did not significantly alter anxiety-like behaviour or locomotion in a novel environment with a component of stress in these tests.

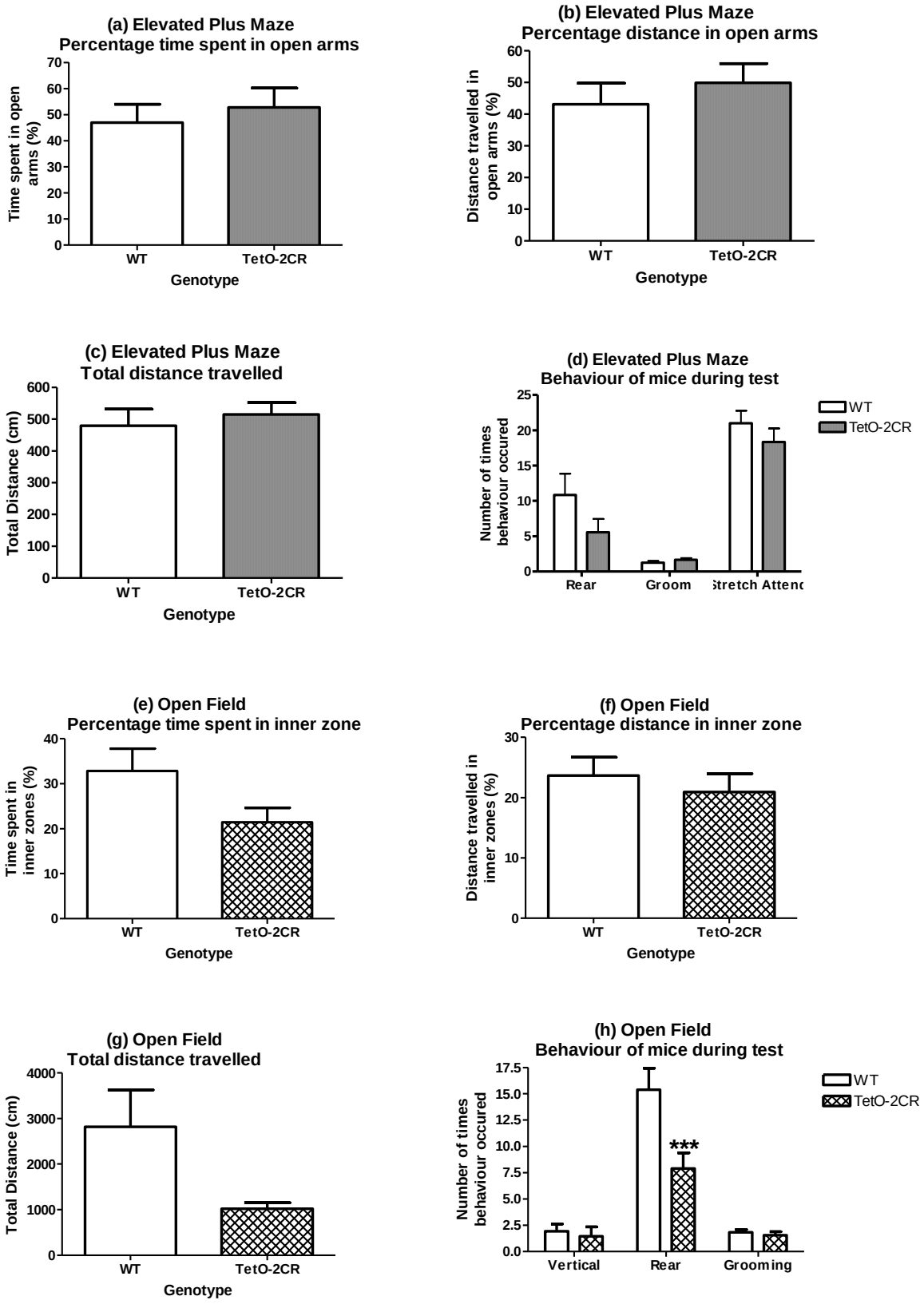


Figure 4.6: Behaviour in the elevated plus maze and open field test in male TetO-2CR mice No alterations were found in the time (a) or distance travelled (b) in the open arms, total distance (c) or behaviour (d) in the EPM. TetO-2CR mice showed trend to reduced time (e) but no alteration in the distance travelled (f) in the inner zone of the OF, with a trend to reduced total locomotion (g) and significantly less rearing behaviour (h) in TetO-2CR mice compared to WT littermate controls. All mice were 12 month old males, backcrossed to C57BL/6 for 5 generations. Data are expressed as mean±SEM and analysed by unpaired Student's t test or 2-way ANOVA with Bonferroni posttests; n=9-12 per group. *** $p<0.001$

The 5-HT_{2C} receptor is implicated in depression, although the literature is conflicting with both agonists and antagonists of this receptor reported to have antidepressant properties (Dunlop *et* al., 2006; Millan *et al*., 2006; Dekeyne *et al*., 2008). One symptom present in many mood disorders, including depression and schizophrenia, is anhedonia, an inability to experience pleasure from normally pleasurable events such as eating (Pelizza & Ferrari., 2009). Sucrose has a natural hedonic effect and reduced consumption of (or preference for) sucrose could be said to indicate an increase in depressive-like behaviour. To test if anhedonic behaviour was altered by 5-HT_{2C} receptor deficiency, the sucrose preference test was used.

The sucrose preference test consists of three sections all with *ad libitum* access to two water bottles. In the first section, both bottles are filled with water (w/w). In the second, both bottles are filled with a sucrose solution (s/s) and during the third section, which is the preference stage, mice are given one bottle filled with water and one filled with the sucrose solution (w/s).

The total volume of liquid drunk differed significantly depending on the stage of the test, with both TetO-2CR and WT mice drinking more during the stage with two sucrose (s/s) bottles (Figure 4.7a: $F_{2,27}$=4.72; p=0.017) but there was no alteration in liquid intake between genotypes (Figure 4.7a: effect of genotype $F_{1,27}$=0.80; p=0.380) or an interaction between the genotype and stage of test (Figure 4.7a: $F_{2,27}$=1.08; p=0.355). TetO-2CR mice did have a significantly greater water intake compared to WT mice (Figure 4.7b; p=0.011) but there was no difference between genotypes in sucrose intake during the preference (w/s) stage (Figure 4.7c; p=0.19).

The preference for sucrose over water is tests hedonic behaviour. Consistent with this no difference in sucrose preference was found between genotypes (Figure 4.7d; p=0.24) suggesting that the deficiency in 5-HT_{2C} receptor in TetO-2CR mice does not alter hedonic behaviour indicating unaltered depressive-like characteristics.

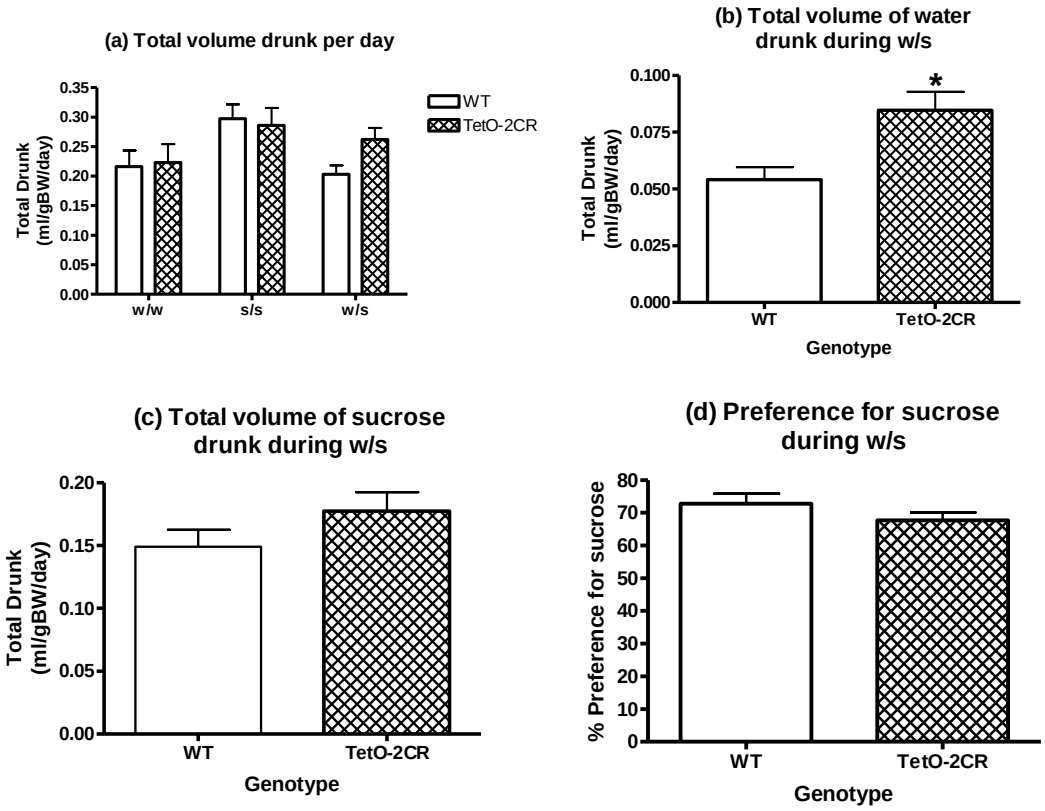


Figure 4.7: Volume drunk and sucrose preference in male TetO-2CR mice during the sucrose preference test

Total volume drunk increases in both genotypes in the s/s stage of the sucrose preference test (a). TetO-2CR mice drink significantly more water than WT during the preference (w/s) stage of the test (b) but both genotypes drink a similar volume of sucrose during the w/s stage if the test (c). There was no difference in sucrose preference between genotypes during w/s stage (d; values calculated by: (vol sucrose/total volume drank)*100). All data is corrected for body weight (BW) and is in TetO-2CR males backcrossed to C57BL/6 for 5 generations. Data are expressed as mean±SEM, , TetO-2CR mice are compared to WT littermate control mice by either 2-way ANOVA with Bonferroni posttests or unpaired Student's t tests; n=6 per group. * $p<0.05$. Two water bottles (w/w), two sucrose bottles (s/s) and one of each during the preference stage (w/s).

4.2.6: 5-HT_{2C} receptor deficiency causes hyperlocomotion and increases agility and balance in TetO-2CR mice

Another phenotype regulated by 5-HT_{2C} receptor is activity/locomotion, with agonists such as mCPP causing hypolocomotion (Gleason *et al.*, 2001). Voluntary wheel running was used to examine the effect of 5-HT_{2C} receptor deficiency in TetO-2CR mice. This allows monitoring of the locomotion of an individual mouse over an indefinite period of time, providing data on the total activity as well as the pattern of wheel running throughout the experiment. During the initial characterisation of the TetO-2CR line, TetO-2CR males showed a trend toward increased wheel running behaviour compared to WT controls although this did not achieve significance (Figure 4.8a; Kimura, Chapman & Holmes unpublished observation; p=0.094), and no alteration in wheel running was found in TetO-2CR mice compared to WT mice following backcrossing to C57BL/6 for 4 generations. However TetO-2CR mice backcrossed for 5 generations appeared to be hyperactive compared to WT mice (Figure 4.8b; genotype $F_{1,19}$=9.69; p=0.006; generation $F_{1,19}$=6.20; p=0.022). There was also an interaction between genotype and the generation of backcrossing (Figure 4.8b; $F_{1,19}$=4.38; p=0.05). This variability in the hyperlocomotor phenotype is primarily due to altered behaviour in WT mice as the strain background changes towards C57Bl/6, with consistent behaviour seen in TetO-2CR mice independent of strain (Figure 4.8b). However, TetO-2CR mice did show a consistent difference in running pattern to WT littermates, with TetO-2CR mice having a consistently later offset (the time at which running stops each day) of running time (Figure 4.8c; genotype $F_{1,25}$=19.58; p=0.0002) in both generation 4 and 5 (effect of generation $F_{1,25}$=0.01; p=0.928) and no interaction of genotype and generation ($F_{1,25}$=0.38; p=0.541) was found.

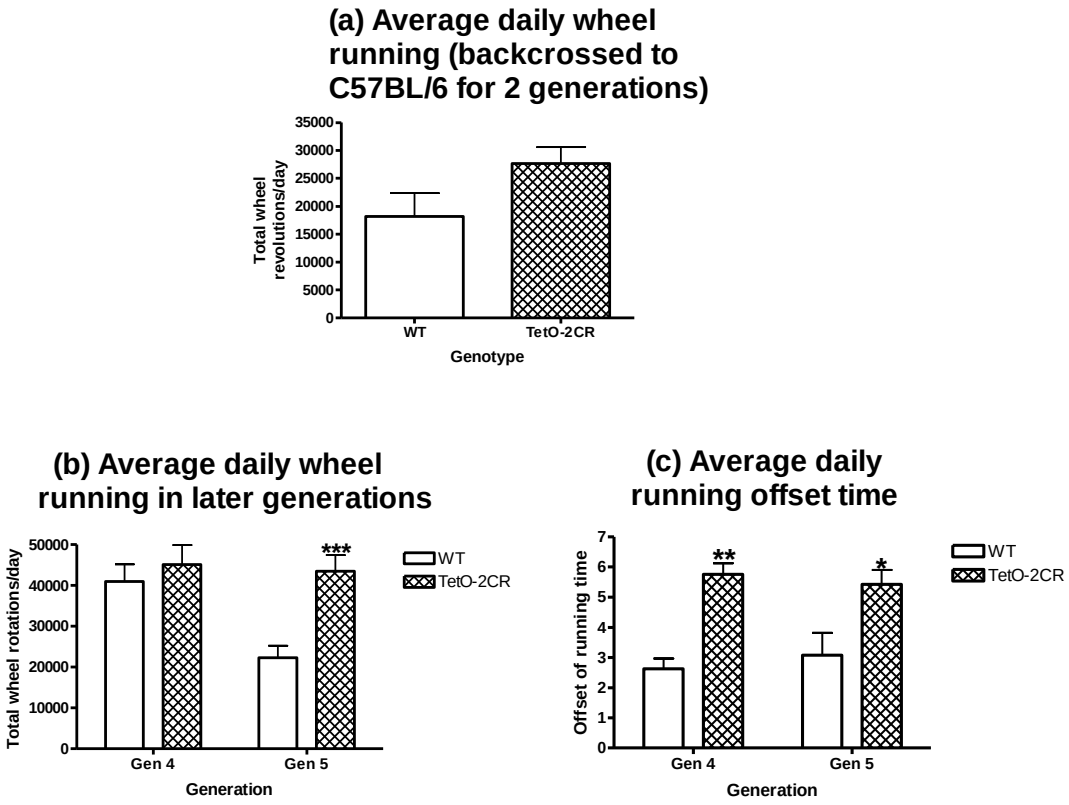


Figure 4.8: Behaviour in wheel cages in male TetO-2CR mice
Following backcrossing to C57BL/6 for 2 generations a trend to increased wheel running was found by A. Kimura in TetO-2CR mice compared to WT mice (a). Following re-derivation at a new animal facility there was no alteration between genotypes backcrossed to C57BL/6 for 4 generations, however a significant increase in wheel running was found in TetO-2CR mice compared to WT mice (b). TetO-2CR mice had a consistently later offset of running time compared to WT mice (c). All mice were individually housed in wheel running cages. Data are expressed as mean±SEM and TetO-2CR mice are compared to WT littermate control mice by unpaired Student's t test (A) and by two-way ANOVAs (B and C); n=6 per group. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

In order to assess the general agility and balance of mice a rotorod test was undertaken. TetO-2CR mice had increased agility and balance compared to WT mice as shown by increased time on the rotorod (Figure 4.9; p=0.027).

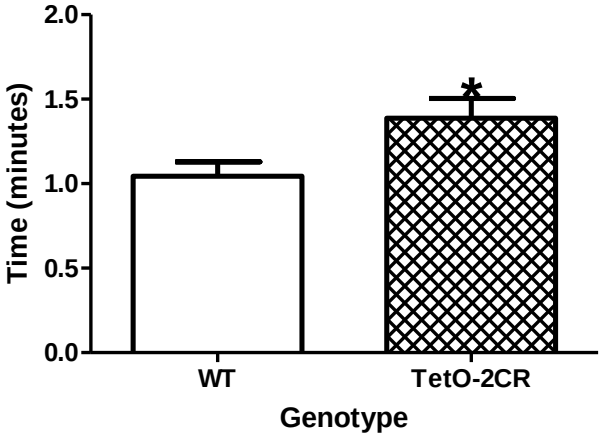


Figure 4.9: Length of time on the rotorod in male TetO-2CR mice TetO-2CR mice are able to stay on the rotorod (without circling or falling off) longer than WT littermate control mice, indicating increased agility and balance in TetO-2CR males, backcrossed to C57BL/6 for 5 generations. Data are expressed as mean±SEM and analysed by unpaired Student's t test; n=12 per group. * p<0.05.

4.2.7 Altered activation of 5-HT_{2C} receptor ligands in TetO-2CR mice

To test the effect of 5-HT_{2C} receptor ligands upon locomotion, male mice were singly housed in cages with wheels.

RO 60-0175, an agonist selective for the 5-HT_{2C} receptor, was administered by intraperitoneal (IP) injection, 30 min prior to the onset of the dark phase (7pm). Injection of 5mg/kg agonist inhibited wheel running behaviour slightly in TetO-2CR mice (~20% inhibition) but had a greater effect on WT mice with ~80% inhibition of

wheel running (Figure 4.10; p=0.002). This slight inhibition in TetO-2CR mice implied that the low number of 5-HT_{2C} receptors provided decreased functionality.

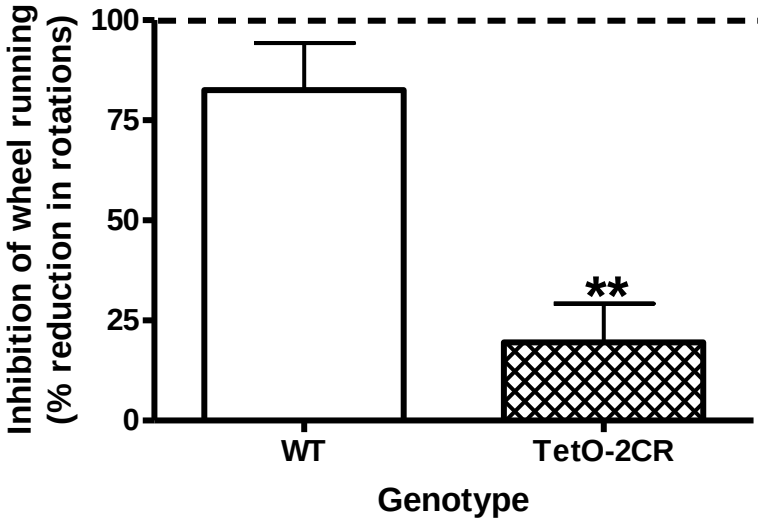


Figure 4.10: Inhibition of voluntary wheel running following administration of RO 60-0175 in male TetO-2CR mice
Inhibition of wheel running is greater in WT mice compared to TetO-2CR mice following IP injection of 5mg/kg RO 60-0175. All mice were backcrossed to C57BL/6 for 3 generations. Data are expressed as mean±SEM and TetO-2CR mice are compared to WT littermate control mice by unpaired Student's t test; n=6. ** p<0.01.

SB206553 is a selective 5-HT_{2C} receptor inverse agonist in rats, however when tested in 5-HT_{2C} receptor over-expressing transgenic mice (C2CR.10 and C2CR.33; Chapter 3) it caused an inhibition of locomotor activity rather than the anticipated increase. When SB 206553 was administered intraperitoneally to TetO-2CR and WT mice in wheel cages 30 min prior to onset of dark phase (7pm), there was inhibition of wheel running in both genotypes with no difference in response between the genotypes (Figure 4.11; p=0.818), suggesting that this effect was not

solely on the 5-HT_{2C} receptor (TetO-2CR mice having significantly reduced receptor number and functionality).

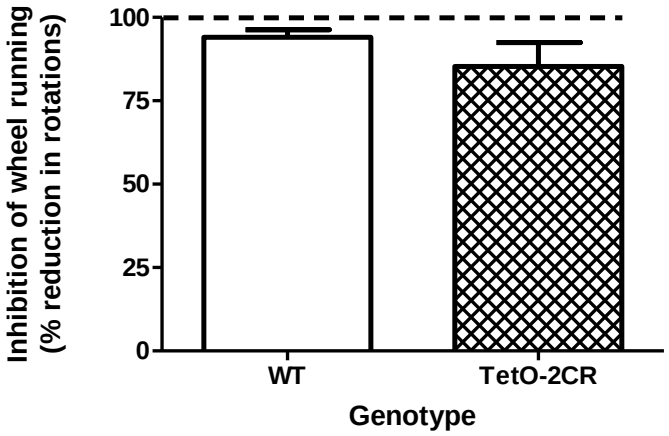


Figure 4.11: Inhibition of voluntary wheel running following administration of SB 206553 in male TetO-2CR mice
Wheel running was inhibited in both genotypes following IP administration of a 5 mg/kg dose of inverse agonist (SB206553). All mice were backcrossed to C57BL/6 for 3 generations. Data expressed as mean±SEM and TetO-2CR mice are compared to WT littermate control mice by unpaired Student's t test; n=6 per group.

4.2.8 Altered response to a novel environment in TetO-2CR mice

TetO-2CR mice, when they were about to be handled, would twirl their tails rapidly and become defensive. Their behaviour made them hard to handle for injections. During voluntary wheel running experiments TetO-2CR mice would also need a longer acclimatisation period to settle into a consistent wheel running rhythm than WT mice.

4.3 Effect of 5-HT_{2C} receptor deficiency on LEO and FEO regulation in TetO-2CR mice

4.3.1: 5-HT_{2C} receptor deficiency causes no alteration in circadian food or water consumption in TetO-2CR mice

As mentioned previously, the 5-HT_{2C} receptor is expressed in a circadian manner in rat (Holmes *et al*., 1997) and plays a role in regulating food intake with 5-HT_{2C} receptor KO mice being hyperphagic (Tecott *et al*., 1995), with the deficiency in 5-HT_{2C} receptor in TetO-2CR mice causing hyperphagia. To investigate if TetO-2CR mice have a different circadian eating pattern to WT mice, food and drink intake were monitored continuously over a 24 hr period. The influence of the LEO on food intake was also examined by monitoring under normal lighting conditions (LD, lights on 0700, off 1900) and in constant darkness (DD).

Although TetO-2CR mice consumed more calories than WT mice in both lighting conditions (Figure 4.12a; effect of genotype $F_{1,140}$=8.80; p=0.004; effect of lighting condition $F_{1,140}$=0.65; p=0.421), their circadian pattern of eating was similar to WT mice (Figure 4.12b/c).

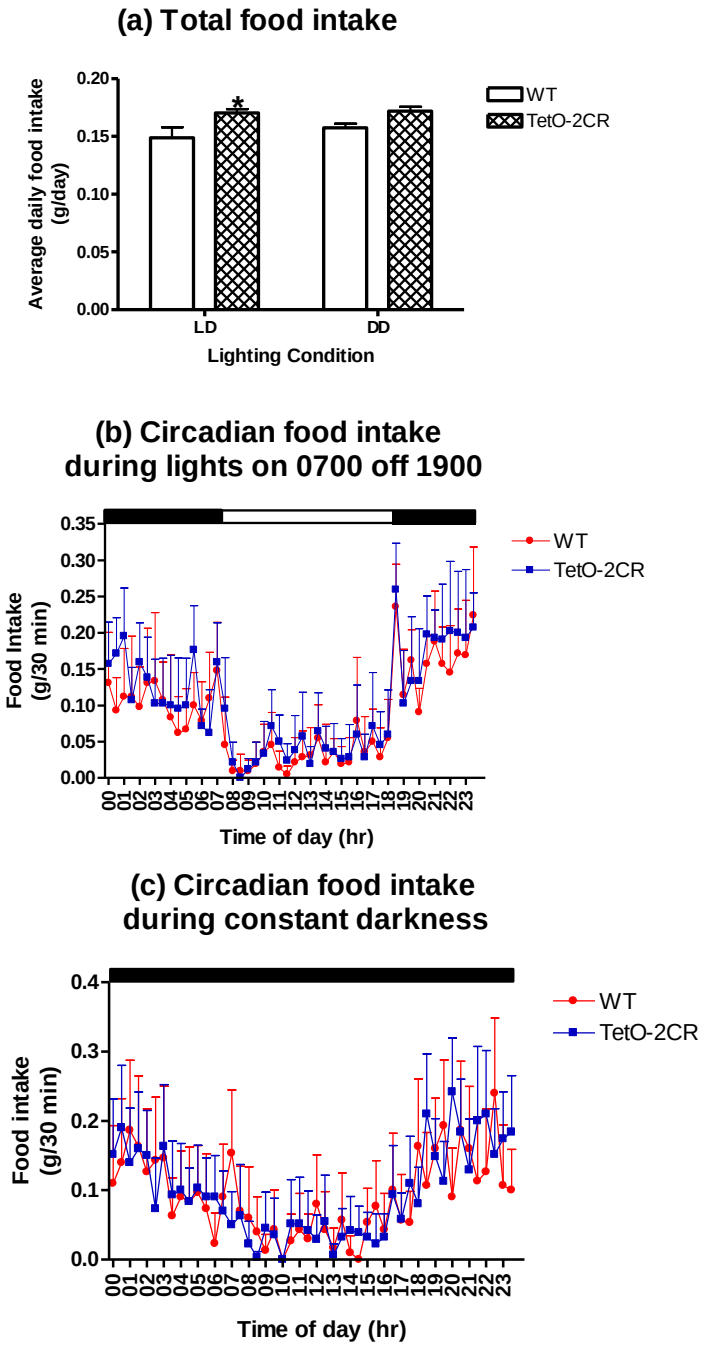


Figure 4.12: Circadian food intake in male TetO-2CR mice
Total food intake per day (corrected for BW) was increased in TetO-2CR mice compared to WT mice during LD (lights on 0700 off 1900) with a significant effect of genotype but no effect of the lighting condition (a). No alteration in circadian eating pattern (g/30 minute not corrected for BW) was found between genotypes in LD conditions (b) or DD condition (c; lights continuously off). All data were collected in male mice backcrossed to C57BL/6 for 4 generations, aged 3 months. Lights on represented by white box and lights off represented by black box. Data are expressed as mean±SEM and TetO-2CR mice compared to WT littermate control mice by two-way ANOVA with Bonferroni posttests; n=6 per group. * $p<0.05$.

In LD conditions both genotypes have a similar water consumption (Figure 5.18).

During In LD conditions both genotypes have similar water consumption (Figure 4.13). During DD conditions WT mice drank more compared to LD (effect of lighting condition $F_{1,92}$=4.91; p=0.029) and so in DD conditions TetO-2CR mice have a lower volume of water drunk compared to WT mice (effect of genotype $F_{1,92}$=4.86; p=0.03).

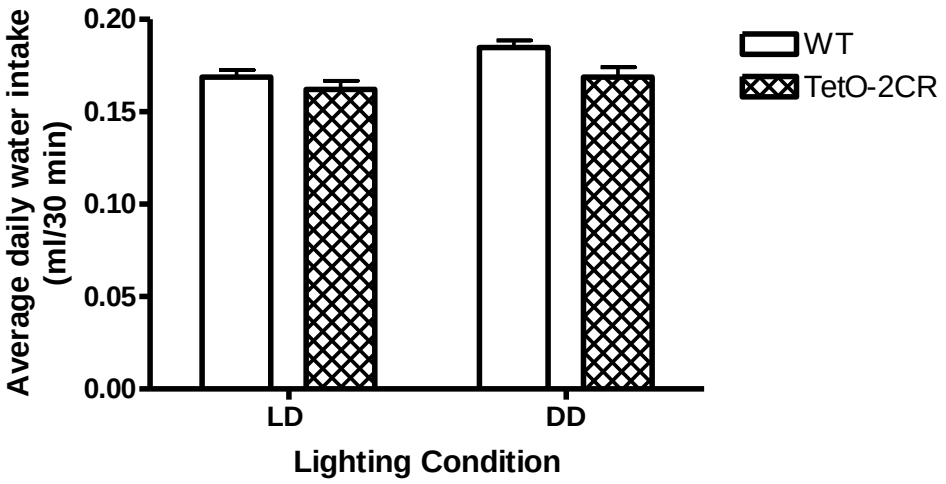


Figure 4.13: Water intake in altered lighting conditions in male TetO-2CR mice Total water intake (corrected for BW) was similar between genotypes in LD conditions (lights on at 0700 and off at 1900). WT mice drink more during DD (constant darkness) than during LD conditions and so in DD conditions WT mice drink more than TetO-2CR mice. All mice are males, backcrossed to C57BL/6 for 4 generations. Data are expressed as mean±SEM and TetO-2CR mice are compared to WT littermate control mice by two-way ANOVA with Bonferroni posttests; n=6 per group.

4.3.2 5-HT_{2C} receptor deficiency does not alter regulation of the food entrainable oscillator (FEO) in TetO-2CR mice

The food entrainable oscillator (FEO), although its location is still unknown, enables the animal to entrain to food availability. When animals are fed at one time point each day (the same time each day) they show food anticipatory (FA) running

behaviour prior to food presentation, even when the SCN is lesioned. When food is presented outside the normal activity period the circadian rhythm regulated by the LEO and that of the FEO are dissociated. This results in two distinct behavioural components which can be monitored; the normal nocturnal activity regulated by the master clock and entrained by the LEO and food anticipatory running behaviour regulated by the FEO.

To test the regulation of the FEO in TetO-2CR mice, food availability was gradually reduced over a week to a period of 6 hrs during the light phase. Throughout the experiment, TetO-2CR mice maintained a higher body weight (Figure 4.14a; $F_{1,30}$=68.22; p<0.0001) as well as an increased food intake (Figure 4.14b; $F_{1,30}$=9.49; p=0.004) compared to WT mice which doesn't reach significance during LD conditions but is significantly increased in DD conditions during food restriction.

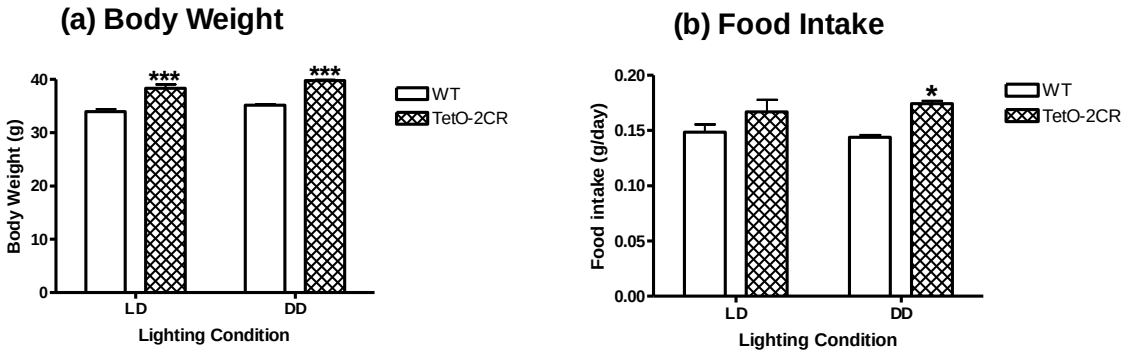


Figure 4.14: Body weight and food intake in male TetO-2CR mice during food restriction in wheel cages

TetO-2CR mice are heavier than WT mice during food restriction in both LD and DD conditions (a). Total food intake per day (corrected for BW) TetO-2CR mice have increased daily food intake (corrected for BW) during food restriction (b; food available 11am to 5pm). All results generated using males, backcrossed onto C57BL/6 for 3 generations, 10 months of age. Data are expressed as mean±SEM and TetO-2CR mice are compared to WT littermate control mice using 2-way ANOVA with Bonferroni posttests; n=6 per group. * p<0.05, *** p<0.001.

Both WT and TetO-2CR mice exhibited food anticipatory running behaviour (Figure 4.15a and b). Imposition of constant darkness (DD) causes the onset of running to drift slightly each day as the mice are no longer able to entrain their running pattern to light cues and this is termed a free-running rhythm. As would be anticipated WT showed a strong free running rhythm, but TetO-2CR mice running did not and ran more sporadically. Interestingly in DD conditions, three of the TetO-2CR mice ran throughout the period of time when food was available, however they managed to maintain their body weight (Figure 4.15b, Tet 2 is a typical example). Throughout the test a trend to increased wheel running is found in TetO-2CR mice compared to WT mice regardless of light condition during the food restriction (Figure 4.15c; $F_{1,20}$=3.65; p=0.07). Running during Light phase of LD conditions similar between TetO-2CR and WT mice indicating similar levels of food anticipatory running behaviour (Figure 4.15d; p=0.91).

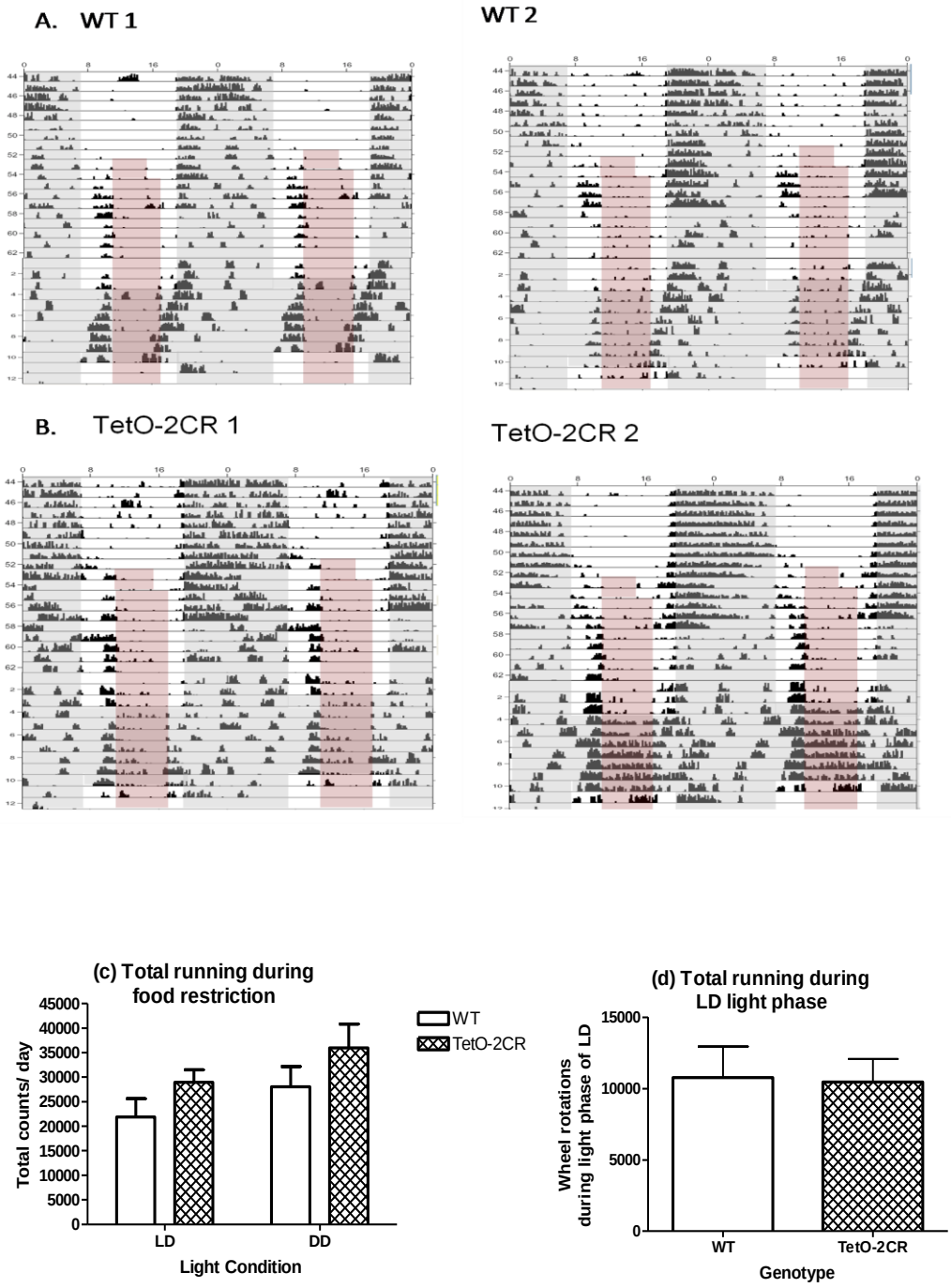


Figure 4.15: Behaviour during food restriction in wheel cages in male TetO-2CR mice
Example of typical WT (a) and TetO-2CR mice (b) double plotted actograms (2 cycles per line with the second cycle on a line being the same as the first cycle on the next line) during food restriction experiment. Grey shading represents periods when lights are off and pink shading representing times when food was available. Total running per day during food restriction is slightly increased in TetO-2CR mice compared to WT mice in both LD and DD conditions (c). Daily running during the light phase of LD was unaltered between genotypes (d). All mice were males, backcrossed to C57BL/6 for 5 generations. Data are expressed as mean±SEM and TetO-2CR mice compared to WT littermate control mice by two-way ANOVA with Bonferroni posttests; n=6 per group.

It was concluded that deficiency in the 5-HT_{2C} receptor does not alter the regulation of the FEO, although there was an indication that alterations may be found in the LEO regulation due to the lack of a strong free running rhythm in DD conditions in TetO-2CR mice. An addition to this experiment should be a comparison of food consumption in both lighting condition during *ad lib* and food restriction.

4.3.3 Effect of 5-HT_{2C} receptor deficiency on the light entrainable oscillator (LEO)

The LEO is located within the SCN and synchronises the endogenously generated circadian rhythm with the exogenous light dark cycle. A role for the 5-HT_{2C} receptor in regulating the LEO has recently been suggested as 5-HT_{2C} receptor agonists given in the first half of the dark phase induced clock gene expression of *Per*1 and *Per*2 which are essential for rhythm responses to external stimuli such as light. It is already established that the SCN receives serotonergic input from the raphe nucleus and that 5-HT_{2C} receptor is expressed in the SCN (Varcoe & Kennaway., 2008).

In order to assess if the 5-HT_{2C} receptor plays a role in the regulation of the LEO, individual wheel running activity was monitored in different light conditions (Figure 4.16). Following a period of acclimatisation, lighting was altered from normal LD (on at 0700, off at 1900) to 3-3 (on at 0300, off at 1500) to asses ability to entrain and finally mice were put into constant darkness (DD) to examine free running rhythm before reimposition of the normal LD conditions.

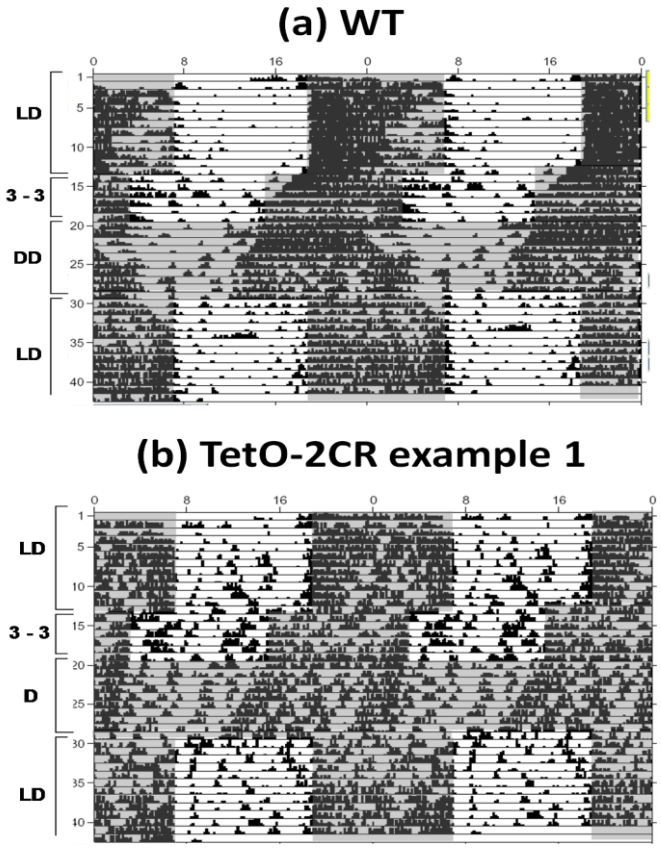


Figure 4.16: Effect of light alterations on voluntary wheel running behaviour in male TetO-2CR mice

Example of a typical WT mouse (a) and the two typical running patterns generated by TetO-2CR mice (b) in double plotted actograms (2 cycles per line with the second cycle on a line being the same as the first cycle on the next line). All mice are males, backcrossed to C57BL/6 for 4 generations. Grey shading represents when lights are switched off. LD (on at 0700, off at 1900) to 3-3 (on at 0300, off at 1500) and finally constant darkness (DD).

TetO-2CR mice (backcrossed to C57BL/6 for 3 generations) ran more than WT mice during LD and DD conditions, although following a phase advance to 3-3 conditions there was no difference between genotypes (Figure 4.17a; genotype $F_{1,60}$=4.97; p=0.03) and no effect of lighting condition ($F_{2,60}$=0.40; p=0.669) or an interaction between genotype and lighting condition was found ($F_{2,60}$=0.72; p=0.49). TetO-2CR mice (backcrossed to C57BL/6 for 5 generations) ran more than WT mice in 3-

3 condition but no differences were found between genotypes in LD or DD conditions (Figure 4.17b; $F_{1,16}$=3.92; p=0.065) and no effect of lighting condition ($F_{2,16}$=1.19; p=0.331) or an interaction of genotype with lighting condition was found ($F_{2,16}$=0.65; p=0.538).

The offset time of running was significantly later in TetO-2CR mice compared to WT mice backcrossed to C57BL/6 for 3 generations in all lighting conditions with a significant effect of genotype (Figure 4.17c; $F_{1,203}$=315.72; p<0.0001), lighting condition ($F_{2,203}$=178.60; p<0.0001) and the interaction of genotype with lighting condition ($F_{2,203}$=240.11; p<0.0001). Following backcrossing to C57BL/6 for 5 generations the offset in TetO-2CR mice was still later than WT mice in LD and 3-3 conditions although no difference was found between genotypes in DD conditions (Figure 4.17d; genotype effect $F_{1,156}$=14.61; p=0.0002) and a significant effect of lighting condition ($F_{2,156}$=105.91; p<0.0001) and the interaction of genotype and lighting condition were found ($F_{2,156}$=39.46; p<0.0001). Interestingly when lighting conditions were phase advanced to the 3-3 condition TetO-2CR mice maintained their original offset time from the LD conditions, while WT mice moved their offset time dependent on the lighting conditions in both generation 3 and 5. Generation 3 TetO-2CR mice maintained this pattern of running following imposition of constant darkness, with their offset time remaining around 7am (Figure 4.17c). The offset time in generation 5 TetO-2CR mice shifted to being similar to WT mice in constant darkness (Figure 4.17d).

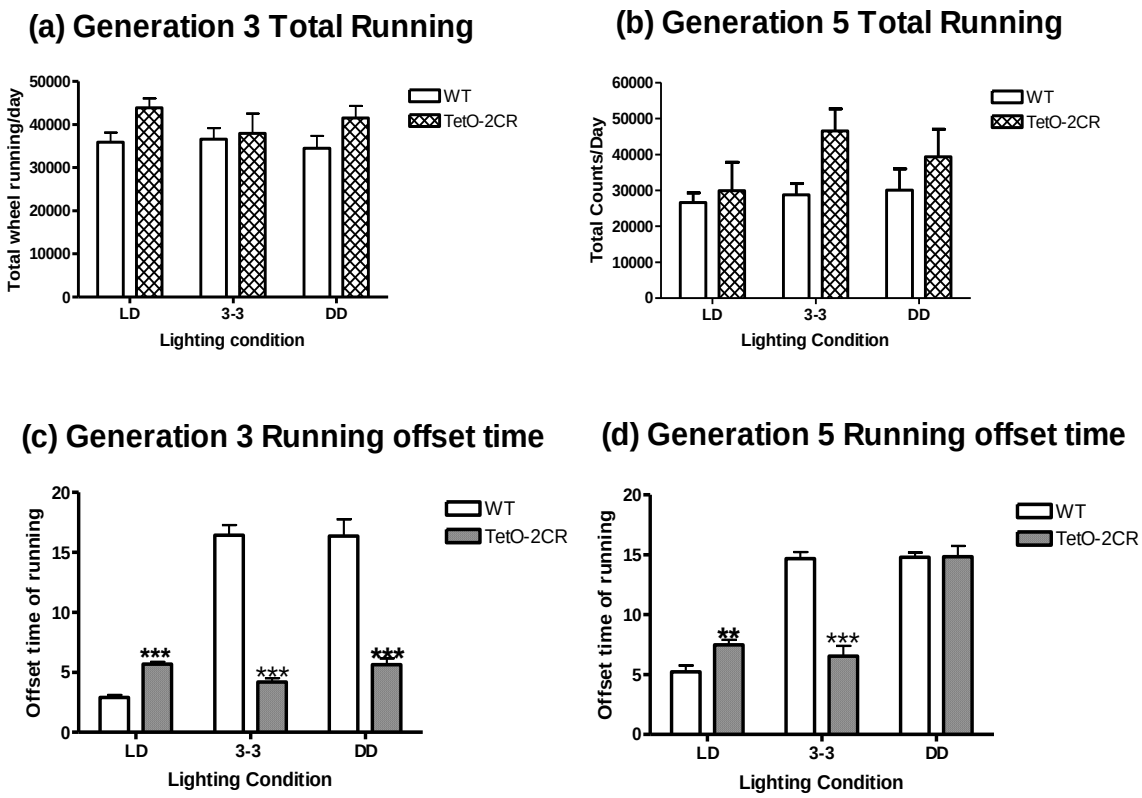


Figure 4.17: Effect of light alterations on voluntary wheel running and running offset time in male TetO-2CR mice

TetO-2CR mice have increased wheel running compared to WT mice in LD conditions following backcrossing to C57BL/6 for 3 generations (Figure 5.22a) but no difference was found between genotypes following backcrossing to C57BL/6 for 5 generations (Figure 5.22b). Running was similar between genotypes in generation 3 but increased in TetO-2CR mice in generation 5 during 3-3 conditions. In response to the imposition of constant darkness generation 3 and 5 TetO-2CR mice ran slightly more than WT mice. The offset of running time was significantly later in TetO-2CR mice compared to WT mice during LD and 3-3 conditions following backcrossing to C57BL/6 for 3 and 5 generations (c, d). Mice were individually housed in wheel cages and their activity monitored following an acclimitisation period under three different lighting conditions, LD (lights on 0700 and off 1900), 3-3 (lights on 0300 and off 1500) and DD (constant darkness). Data are expressed as mean±SEM and TetO-2CR mice are compared to WT littermate control mice by two-way ANOVA with Bonferroni posttests; n=6 per group. ** p<0.01, *** p<0.001.

Increased number of running bouts were found in TetO-2CR mice backcrossed to C57BL/6 for 3 generations compared to WT mice in LD and DD conditions but not in 3-3 conditions (Figure 4.18a; effect of genotype $F_{1,295}$=27.42; p<0.0001), there was a trend of an effect of lighting condition ($F_{2,295}$=2.94; p=0.055) and a significant interaction between genotype and lighting condition ($F_{2,295}$=4.04; p=0.019). In TetO-2CR mice backcrossed to C57BL/6 for 5 generations, increased running bouts were also found in LD and DD condition compared to WT mice but not in 3-3 conditions (Figure 4.18b; effect of genotype $F_{1,306}$=86.31; p<0.0001), there was no effect of lighting condition ($F_{2,306}$=1.58; p=0.209) but there was an interaction of genotype with lighting condition ($F_{2,306}$=9.33; p=0.0001).

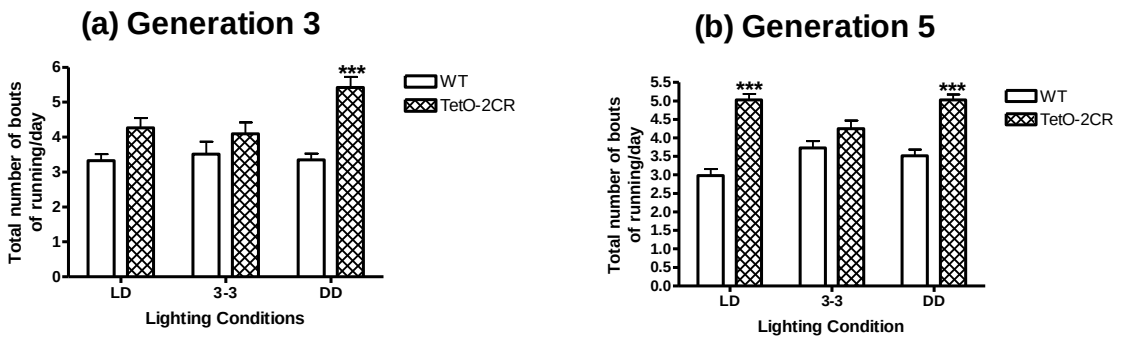


Figure 4.18: Effect of light alterations on the number of wheel running bouts in male TetO-2CR mice

The number of running bouts each day were higher in TetO-2CR mice compared to WT mice in LD conditions and DD conditions but no difference was found between genotypes in 3-3 conditions after backcrossing to C57BL/6 for 3 and 5 generations (a, b). Mice were individually housed in wheel cages and their activity monitored following an acclimitisation period under three different lighting conditions, LD (lights on 0700 and off 1900), 3-3 (lights on 0300 and off 1500) and DD (constant darkness). Data are expressed as mean±SEM and TetO-2CR mice are compared to WT littermate control mice by two-way ANOVA with Bonferroni posttests; n= 6 per group. *** p<0.001.

To further investigate if 5-HT_{2C} receptor deficiency was able to effect the regulation of the LEO a series of light pulses were given in the first half of the dark phase on intermittent days. The average inhibition of wheel running caused by the light pulses was significantly reduced in TetO-2CR mice compared to WT mice (Figure 4.19; effect of genotype; $F_{1,24}$=4.81; p=0.038) and the time the light pulse was given was important ($F_{2,24}$=7.24; p=0.004).

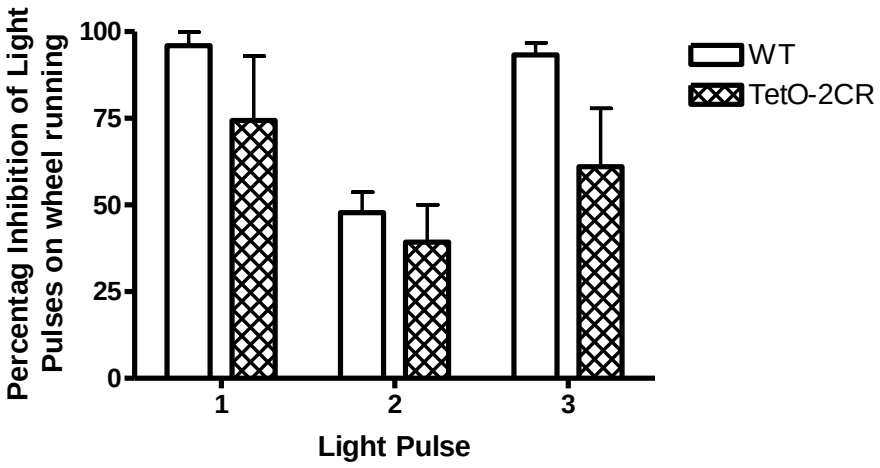


Figure 4.19: The percentage of inhibition on wheel running during light pulses
There were indications that wheel running in TetO-2CR mice was less affected by light pulses than WT mice (pulse 1 = 2000 to 2200, pulse 2= 2100 to 2200, pulse 3 = 2100 to 2300). All mice were males, backcrossed to C57BL/6 for 5 generations. Data are expressed as mean±SEM; TetO-2CR mice were compared to WT littermate control mice by two-way ANOVA with Bonferroni posttests and an unpaired Student's Ttest; n=5-6 per group.

4.4 Discussion

Complete loss of 5-HT_{2C} receptor function leads to a lowered seizure threshold and a more rapid progression of seizure activity, with 5-HT_{2C} receptor knock-out mice (5-HT_{2C} receptor KO mice) suffering from spontaneous seizures from the fifth postnatal week, resulting in death in hemizygous mutant male mice, with a 60% survival rate by 25 weeks (Tecott *et al*., 1995). In TetO-2CR mice no evidence of seizure activity was found, indicating that the low levels of 5-HT_{2C} receptor present during development were sufficient to prevent lowering of seizure threshold. The fact that the TetO-2CR model does not suffer from spontaneous seizures, possibly due to residual function, provides an advantage over the complete 5-HT_{2C} receptor KO mouse.

Quantification showed that 5-HT_{2C} receptor mRNA levels were significantly reduced throughout the brain in TetO-2CR mice compared to WT mice. As the expression of this receptor is driven by the normal 5-HT_{2C} receptor promoter in TetO-2CR mice, they are deficient in 5-HT_{2C} receptor in all regions that the 5-HT_{2C} receptor would normally be found and this reduction is in a region specific manner. The decrease found in 5-HT_{2C} receptor mRNA levels will cause a decrease at the protein level in this model and this has been confirmed using the ^{3}H-mesulergine binding assay to detect 5-HT_{2C} receptor binding. Due to the decreased expression of the 5-HT_{2C} receptor in hypothalamic and limbic regions of TetO-2CR mice, an alteration in food intake, mood and locomotion were anticipated.

The 5-HT_{2C} receptor has been implicated in the regulation of food intake (Chapter 1) with non-selective agonists, such as mCPP, causing advancement in satiety and reducing food intake while specific 5-HT_{2C} receptor antagonists are able to block these anorectic effects (Clifton *et al*., 2000 256-267; Kennett & Curzon., 1988 93-100; Hewitt *et al*., 2002; Samanin 1979 159-163). 5-HT_{2C} receptor KO mice were

found to be hyperphagic and both plasma glucose levels and insulin were similar in KO compared to WTs when on a standard paired feeding analysis (Tecott *et al*., 1995). Due to larger white fat stores, 5-HT_{2C} receptor KO mice develop obesity in later life but no alteration in the level of brown adipose tissue was found compared to WT. This hyperphagia and obesity is due to behavioural abnormalities as opposed to metabolic defects (Tecott *et al*., 1995). Similar to the 5-HT_{2C} receptor KO mice, TetO-2CR mice were hyperphagic and heavier than WT mice by twenty-four weeks of age.

Because food intake is increased by 5-HT_{2C} receptor deficiency and the 5-HT_{2C} receptor is expressed in a circadian manner (Holmes *et al*., 1997) it was considered that the increased food intake may be a consequence of altered regulation of the food entrainable oscillator (FEO). This was, however, not the case as TetO-2CR mice had a similar pattern of food consumption (over a 24 hr period) to WT mice but with increased food intake at each meal. In addition TetO-2CR mice showed food anticipatory running behaviour during a food restriction experiment although moving into constant darkness did increase the difference in food intake between genotypes. I therefore concluded that in this model 5-HT_{2C} receptor deficiency does not alter the regulation of the FEO.

It was hypothesised that anxiety-like behaviour would be decreased in TetO-2CR mice compared to WT mice as the 5-HT_{2C} receptor mRNA levels were decreased throughout limbic regions of the brain which are involved in the alteration of mood. The 5-HT_{2C} receptor plays an important role in the regulation of anxiety-like behaviour with agonists increasing anxiety and antagonists being anxiolytic (Kennett *et al*., 1989; Jenck *et al*., 1998; Harada *et al*., 2008). It has been shown that transgenic mice over-expressing the 5-HT_{2C} receptor in the forebrain showed a slight increase in anxiety-like behaviour (Chapter 3; Kimura *et al*., 2009) while 5-HT_{2C} receptor KO mice had an anxiolytic phenotype (Heisler *et al*., 2007b). Another aspect of mood examined was depressive-like behaviour. The literature on the effects

of agonists and antagonists is conflicting with the majority of papers finding that antagonists have antidepressant properties (Millan *et al*., 2006; Calcagno *et al*., 2008; Dekeyne *et al*., 2008) but there is evidence that 5-HT_{2C} receptor agonists may also be efficacious antidepressants (Dunlop *et al*., 2006). In TetO-2CR mice, however, no strong phenotype was found in tests of anxiety-like or depressive-like behaviour. Following backcrossing of the 5-HT_{2C} receptor over-expressing transgenic mice to C57BL/6, the anxiety-like phenotype was lost and similarly, 5-HT_{2C} receptor KO mice investigated more recently show no difference in anxiety-like or depressive-like behaviours (Hill *et al*., 2010). This indicates that alterations in 5-HT or other systems may have occurred, to compensate for the increased or decreased 5-HT_{2C} receptor levels, and normalise behaviour. The first anxiety tests were undertaken in TetO-2CR mice following several more generations of backcrossing onto C57BL/6 compared to the first tests carried out in the 5-HT_{2C} receptor over-expressing mice and so if TetO-2CR mice had been tested earlier in the backcrossing process it is possible that anxiety-like behaviour might have been altered. It is possible that on the C57BL/6 genetic background anxiety traits are undetectable in both the 5-HT_{2C} over-expressing mice and TetO-2CR mice. Interestingly TetO-2CR mice appear to be hyper-sensitive to novel environments as they took longer to acclimatise to wheel cages compared to WT mice and behaved in a more aggressive manner when handled, particularly when being scruffed. This contradicts the hypothesis that reduced 5-HT_{2C} receptor expression would reduce anxiety-like behaviour.

It will be important to examine BNDF levels in TetO-2CR mice as both the production (Alter 1992) and expression (Nibuya *et al*., 1995) of BDNF in the brain are altered in response to antidepressant treatment. In addition BDNF infusions in rats produce antidepressant effects (Siuciak *et al*., 1997; Shirayama *et al*., 2002) possibly through altering activity of 5-HT, DA and/or NA pathways (Siuciak *et al*., 1997) which are known to be altered in depression. In 5-HT_{2C} receptor KO mice, BDNF levels were elevated possibly contributing to the loss of the anxiety-like and depressive-like phenotype (Hill *et al*., 2010). This is an important area to examine in

TetO-2CR mice as BDNF levels could be elevated and could be a possible mechanism to normalise mood-related behaviours.

The role of 5-HT_{2C} receptor, in regulation of locomotion is well established, with agonists such as mCPP causing motor depression (Fone *et al*., 1998; Gleason *et al*., 2001) and hypolocomotion occurring in transgenic mice over-expressing 5-HT_{2C} receptors in the forebrain (Kimura *et al*., 2009). Similar to 5-HT_{2C} receptor KO mice, which are hyperactive, TetO-2CR mice were more active than WT mice, and consistently showed a later offset time of running in wheel cages. TetO-2CR mice also had increased agility and balance on the rotorod concurring with the hyperlocomotor phenotype. There may be alterations in the DA system which could play a role in the locomotor phenotype and will be discussed in Chapter 5.

One possible candidate to consider is brain-derived neurotrophic factor (BDNF) as it has been linked to locomotor activity (Kernie *et al*., 2000) although data are conflicting regarding whether increased BDNF levels (Sauer *et al*., 1993; Li *et al*., 2007) or decreased levels in BDNF KO mice, (Rios *et al*., 2001; Monteggia *et al*., 2004) account for hyperlocomotor activity while another study reports no alteration in locomotion following selective loss of BDNF in the CA1 or DG (Adachi *et al*., 2008). It is possible that BDNF levels may be altered in TetO-2CR mice as BDNF levels have recently been shown to be elevated in the hippocampus of 5-HT_{2C} receptor KO mice (Hill *et al*., 2010).

5-HT_{2C} receptors are important in sleep regulation with 5-HT_{2C} receptor KO mice having disrupted sleep-wake periods and longer periods of wakefulness (Frank *et al*., 2002). Treatment with agomelatine, a melatonin agonist and 5-HT_{2C} receptor agonist, improves sleep disturbances and is a potential treatment for SAD (Millan *et al*., 2005; Pandi-Perumal *et al*., 2009). Melatonin regulates circadian behaviour and

C57BL/6 mice do not have active melatonin. As previously mentioned the 5-HT_{2C} receptor is involved in depression and the internal clock is profoundly disturbed by depression (Mendlewicz., 2009). It is possible that 5-HT_{2C} receptors could influence the circadian control of sleep through regulation of the LEO. It is already established that 5-HT is one of the neurotransmitters with the ability to phase-advance the LEO via non-photic input pathways (Prosser *et al*., 1990; Kohler *et al*., 1999; Kalkowski & Wollnik., 1999), with various 5-HT receptors suggested to be involved (Kennaway *et al*., 1996; Gannon *et al*., 2009). A role for the 5-HT_{2C} receptor in regulating the LEO has been suggested as 5-HT_{2C} receptor agonists given in the first half of the dark phase (Varcoe *et al*., 2003) induced clock gene expression of *Per*1 and *Per*2 which are essential for rhythm responses to external stimuli such as light (Albrecht *et al*., 1997; Miyake *et* al., 2000; Asai *et al*., 2001; Yan & Silver., 2002). The lack of a free running rhythm in TetO-2CR mice during the DD phase of the food restriction experiment indicated that regulation to the LEO may be altered by the deficiency in 5-HT_{2C} receptors. However, both TetO-2CR mice and WT mice altered their running behaviour similarly in response to alterations in the lighting condition. In one of the trials the offset of running time in TetO-2CR mice did not alter as much as WT mice during a phase advance in lighting condition. The deficiency in 5-HT_{2C} receptors also caused a more "broken-up" pattern of running in TetO-2CR mice compared to WT mice with running becoming more disjointed as lighting conditions progressed to DD. This could be due to a more disrupted sleep wake-cycle, similar to that found in 5-HT_{2C} receptor KO mice.

The 5-HT_{2C} receptor deficiency in TetO-2CR mice may generate differences in behaviour during light alterations that were too subtle to be detected as it has been suggested that the 5-HT_{2C} receptor may regulate the LEO via a secondary pathway (Varcoe *et al*., 2003). This suggested secondary pathway is proposed to project from the retina to raphe nucleus (Shen *et al*., 1994; Fite *et al*., 1999) and then to the SCN. 5-HT is the neurotransmitter that is thought to be utilised in this secondary pathway as 5-HT inputs into the SCN from the raphe nucleus (Moga & Moore., 1997; Hay-Schmidt *et al*., 2003), and 5-HT_{2C} receptors are proposed to be involved as they are

present postsynaptically in the SCN (Moyer & Kennaway., 1999; Clemett *et* al., 2000; Varcoe & Kennaway., 2008). The retinohypothalamic tract (RHT), which utilises glutamate, is the main pathway that enables photic stimuli to be relayed to the SCN (Abe *et al*., 1991). Following blockade of NMDA receptors (therefore blocking the actions of glutamate and the RHT) rats were able to respond to light alterations only in the first half of the dark phase (Rowe *et al*., 1996; Kennaway *et al*., 2001). The RHT could be utilizing another transmitter. However, it is also possible that the system is responding through the secondary pathway (retina to raphe nucleus to SCN), involving 5-HT and the 5-HT_{2C} receptor. Expression of 5-HT_{2C} receptors is on the outer layer of the SCN which puts it in a position to potentially influence circadian entrainment (Moyer & Kennaway., 1999). As previously mentioned a 5-HT_{2C} receptor agonist induced *Per*1 and *Per*2 expression only in the first half of the dark phase (Varcoe *et al*., 2003). There is a suggestion that TetO-2CR mice are less responsive to light exposure in the first half of the dark phase due to the failure of a light pulse given between 2100 and 2300 hours to inhibit running in TetO-2CR mice to the same extent to that found in WT mice. There was also a difference in the response in TetO-2CR mice, compared to WT mice, in running behaviour following administration of the selective 5-HT_{2C} receptor agonist, RO 60-0175. It is suggested that the RHT would need to be blocked in order to establish if a deficiency in the 5-HT_{2C} receptor alters the effectiveness of the suggested secondary pathway.

4.4.1 Concluding remarks

Similar to 5-HT_{2C} receptor KO mice, TetO-2CR mice were hyperphagic and heavier than WT mice in later life. However, TetO-2CR mice have an advantage in that they do not suffer from the spontaneous seizures seen in 5-HT_{2C} receptor KO mice. No alterations were found in anxiety-like or depressive-like behaviour, and in later generations of 5-HT_{2C} receptor KO mice no alterations were found in anxiety-like or depressive-like behaviour. A hyperactive phenotype has been shown in TetO-2CR mice. BDNF is a possible candidate which could normalize behaviours associated

with mood and could have a role in locomotor control and it has recently been shown to be elevated in 5-HT_{2C} receptor KO mice. No alteration in regulation of the FEO was found in TetO-2CR mice however, it is possible that there is an alteration in the regulation of the LEO which may only show significance if the RHT is blocked.

CHAPTER 5:

COMPENSATORY ALTERATIONS IN THE 5-HT SYSTEM AND AFFECTS ON THE DA SYSTEM

Aims

This experimental chapter examines whether there are compensatory alterations in response to altered 5-HT_{2C} receptor expression levels in both the over- and under-expressing mouse lines. The chapter aims were to determine:

1. Compensatory alterations in other 5-HT receptor subtypes
2. Alterations in 5-HT levels in different brain regions
3. Alterations in catecholamine levels

It was hypothesised that compensatory alterations within the 5-HT system may be present in response to altered 5-HT_{2C} receptor levels. A second hypothesis was that the levels of DA would be altered due to increased or reduced inhibition on DA release by increased or reduced levels of 5-HT_{2C} receptor activity.

5.1 Introduction

A phenotype of anxiety and hypolocomotion was initially found in C2CR.10 and C2CR.33 mice which over-express 5-HT_{2C} receptors in the forebrain (see Chapter 3). This behavioural phenotype may have been due to the mixed genetic background of the mice during the initial characterisation (C57BL/6, CBA and C3H) because as the genetic background moved towards congenic C57BL/6, during the backcrossing process, the phenotype disappeared. Another possibility to consider is that aspects of the 5-HT and/or another neurotransmitter system, such as catecholamines, changed to compensate for the increased 5-HT_{2C} receptors, which could normalise behaviour.

5-HT_{2C} receptor agonists increase anxiety-like and depressive-like behaviour and reduce locomotion (Chapter 1 and 3). The regulation of these behaviours is complex and involves a number of other 5-HT receptor subtypes. 5-HT_{1A} receptors and 5-HT_{2A} receptors are two such subtypes which are involved in mood regulation (Gross *et al*., 2000; Millan 2003 70). Alterations in the 5-HT_{1A} receptor can influence 5-HT levels due to its role as an autoreceptor. Changes in 5-HT levels play a role in mood with selective serotonin reuptake inhibitors (SSRIs) used as a treatment for major depression (Deshauer *et al*., 2008). Increased 5-HT levels are associated with decreased locomotor activity.

5-HT_{2C} receptor agonists suppress both dopamine (DA) and noradrenaline (NA) pathways in the frontal cortex (Millan *et* al., 1998; Gobert *et* al., 2000; Di Matteo *et al*., 2001). The DA system plays a role in locomotion, with DA depletion in Parkinson's disease resulting in a loss of movement (Bossy-Wetzel *et al*., 2004). The DA system is implicated in mood disorders with DA system over-activity associated with schizophrenia and 5-HT and NA reuptake inhibitors (SNRIs) are used in the treatment of some anxiety disorders (Dell'Osso*et al*., 2010).

To adapt to alterations in a system (eg. in a KO model), levels of other receptors or neurotransmitters are sometimes found to be altered in order to compensate for lost function and to regain behaviour. It is important to ascertain if altering 5-HT_{2C} receptor levels caused compensatory alterations in the 5-HT and DA systems, which may contribute to any behavioural responses found. The aims of this chapter were therefore to determine if 5-HT_{2C} receptor over-expression or under-expression resulted in (1) compensatory alterations in other 5-HT receptor subtypes; (2) alterations in 5-HT levels in different regions of the brain; and (3) if there was an effect on other catecholamine levels using the 5-HT_{2C} receptor over-expressing (C2CR.10 and C2CR.33 mice; discussed in Chapter 3) and under-expressing mice (TetO-2CR mice; discussed in Chapter 4).

5.2 Results

5.2.1 The Serotonergic System: 5-HT_{1A} receptor mRNA levels

To assess whether expression of other 5-HT receptor subtypes were affected by 5-HT_{2C} receptor over-expression *in situ* hybridisation was used. This technique allowed quantification of 5-HT_{1A} and 5-HT_{2A} receptor mRNA levels in brain sections. The 5-HT_{1A} receptor was chosen due to its roles in anxiety-like behaviour and its role as an autoreceptor of the 5-HT system. Compared to WT mice, C2CR.10 mice had reduced 5-HT_{1A} receptor mRNA which reached significance in the CA1 field of the dorsal hippocampus (Figure 5.1a; effect of genotype $F_{1,30}$=17.61; p=0.0002). C2CR.33 mice had reduced 5HT_{1A} receptor mRNA levels compared to WT mice (Figure 5.1b; effect of genotype $F_{1,40}$=5.41; p=0.025). Interestingly, 5-HT_{1A} receptor mRNA levels were increased in dorsal hippocampus (all regions) as well as amygdala in TetO-2CR mice compared to WT mice (Figure 5.1c; genotype: $F_{1,35}$=25.12; p<0.0001; region: $F_{4,35}$=25.09; p<0.0001) but there was no interaction of the genotype with the region (Figure 5.1c; $F_{4,35}$=1.66; p=0.182).

The 5-HT_{1A} receptor mRNA levels were also examined in the raphe nucleus due to its role as an autoreceptor of the 5-HT system in this region (Figure 5.1d). No alterations between C2CR.33 and WT mice were found in the dorsal or medial raphe nucleus (effect of genotype $F_{1,21}$=0.67; p=0.422).

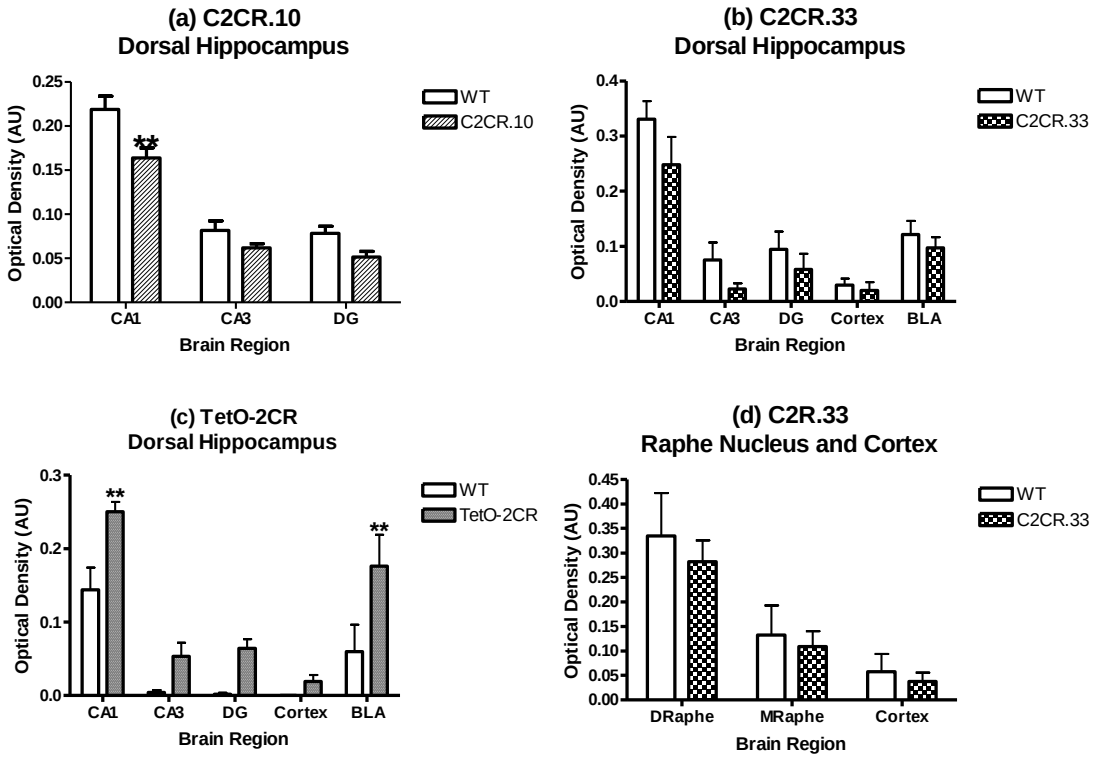


Figure 5.1: 5-HT_{1A} receptor mRNA expression

5-HT_{1A} receptor mRNA levels were decreased in C2CR.10 mice compared to WT mice (a), achieving significance in the CA1 of the dorsal hippocampus. C2CR.33 mice also had reduced 5-HT_{1A} receptor mRNA levels (b) compared to WT mice. 5-HT_{1A} receptor mRNA levels were increased in the dorsal hippocampus (CA1, CA3 and DG), cortex and amygdala (BLA) in TetO-2CR mice (c) compared to WT mice. In the raphe nucleus or cortex (d) there was no difference in 5-HT_{1A} receptor mRNA levels between C2CR.33 and WT mice. Expression levels were measured by optical densitometry of *in situ* hybridisation autoradiographs. All mice were males; C2CR.10 and TetO-2CR mice were backcrossed to C57BL/6 for 2 generations and C2CR.33 mice were backcrossed to C57BL/6 for 7 generations. Data are expressed as mean±SEM; C2CR.10, C2CR.33 or TetO-2CR mice were compared to WT littermate control mice by 2-way ANOVA with Bonferroni posttests; n=5-6 per group. ** $p<0.01$.

CA1 = CA1 field of the hippocampus; CA3 = CA3 field of the hippocampus; DG = dentate gyrus; BLA = basolateral amygdala; DRaphe = dorsal raphe nucleus; MRaphe = median raphe nuclei

5.2.2 5-HT_{2A} receptor mRNA levels

The 5-HT_{2A} receptor is the sister receptor to the 5-HT_{2C} receptor and due to their similarities in structure and due to its involvement in anxiety-like behaviour alterations in this receptor were possible. 5-HT_{2A} receptor mRNA levels in ventral hippocampal regions were unaltered in C2CR.10 mice compared to WT mice (Figure 5.2a; effect of genotype $F_{1,27}$=2.16; p=0.153; interaction of genotype and region $F_{2,27}$=0.17; p=0.846), although levels were higher in the ventral CA3 field of the hippocampus in both genotypes (effect of region $F_{2,27}$=173.91; p<0.0001). C2CR.33 mice had lower 5-HT_{2A} receptor mRNA levels than WT mice (Figure 5.2b; effect of genotype $F_{1,9}$=13.17; p=0.006) generated by a significant decrease in the CA1 field of the ventral hippocampus. In both genotypes, 5-HT_{2A} receptor mRNA levels were increased in the CA3 field of the ventral hippocampus (effect of region $F_{2,9}$=240.06; p<0.0001) but there was no interaction of genotype with region ($F_{2,9}$=1.25; p=0.331). In TetO-2CR mice, 5-HT_{2A} receptor mRNA levels did not differ compared to WT mice in ventral hippocampus or cortex (Figure 5.2c; $F_{1,15}$=1.94; p=0.184), although in both genotypes, levels were higher in ventral CA3 than in ventral CA1 or cortex (Figure 5.2c; $F_{2,15}$=180.82; p<0.0001).

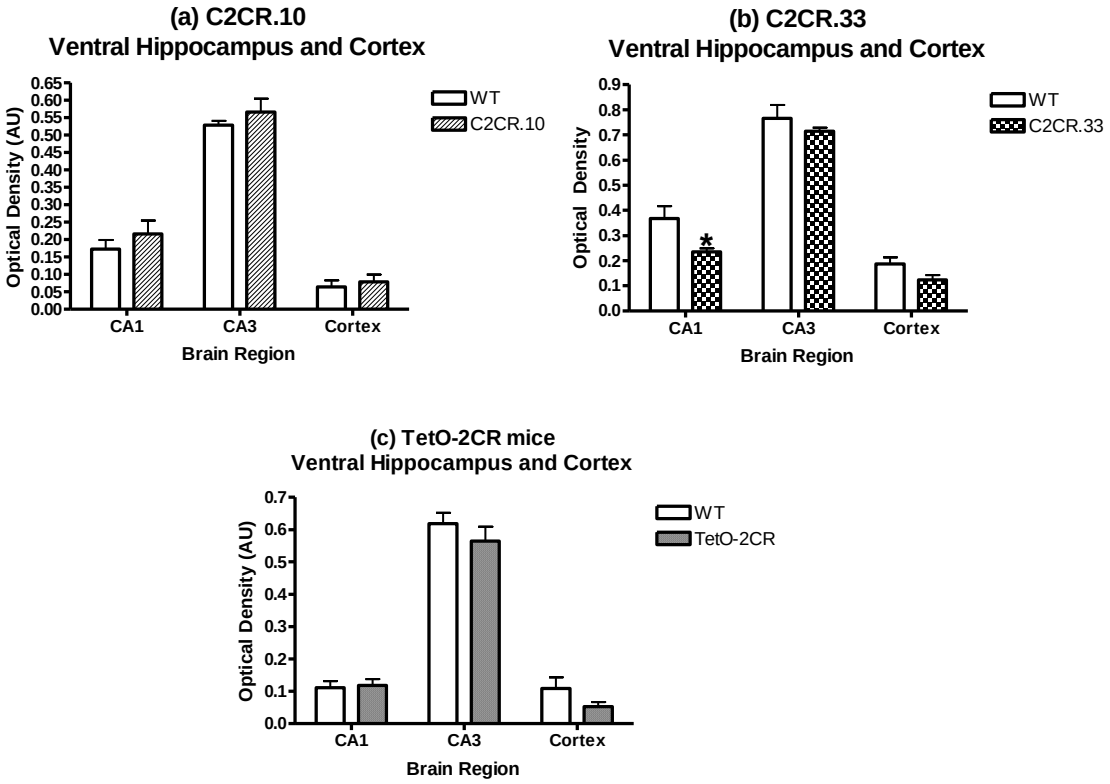


Figure 5.2: 5-HT_{2A} receptor mRNA expression

5-HT_{2A} receptor mRNA levels were unaltered between C2CR.10 and WT mice (a). Mice were males backcrossed to C57BL/6 for 2 generations. C2CR.33 mice had reduced levels of 5-HT_{2A} receptor compared to WT mice (b) reaching significance in the CA1 field of the hippocampus. Mice were males backcrossed to C57BL/6 for 7 generations. 5-HT_{2A} receptor mRNA levels were similar between TetO-2CR and WT mice (c) in ventral hippocampus and cortex with higher levels found in the CA3 of both genotypes. Mice were males backcrossed to C57BL/6 for 2 generations. Expression levels were measured by optical densitometry of *in situ* hybridisation autoradiographs. Data expressed as mean±SEM.. C2CR.10, C2CR.33 or TetO-2CR mice compared to WT littermate control mice by 2-way ANOVA with Bonferroni posttests; n=5-6. * $p<0.05$.

CA1 = CA1 field of the hippocampus; CA3 = CA3 field of the hippocampus

5.2.3 Tryptophan Hydroxylase 2 and 5-HT transporter mRNA levels

Tryptophan hydroxylase (TPH) is the rate-limiting enzyme in the production of 5-HT with TPH type 2 (TPH2) being the relevant form of the enzyme in the brain. Alterations in the mRNA levels of TPH2 could indicate altered activity of this enzyme which could alter the production of 5-HT. In mice backcrossed to C57BL/6 for 7 generations a significant effect of genotype was found with C2CR.33 mice having lower levels of TPH2 compared to WT mice (Figure 5.3a; $F_{1,16}$=4.75; p=0.0447). No alterations in the 5-HT transporter (5-HTT) mRNA levels were found in C2CR.33 mice compared to WT mice, backcrossed to C57BL/6 for 7 generations (Figure 5.3b; effect of genotype $F_{1,12}$=0.01; p=0.9173) indicating that the transportation of 5-HT is similar between C2CR.33 and WT mice.

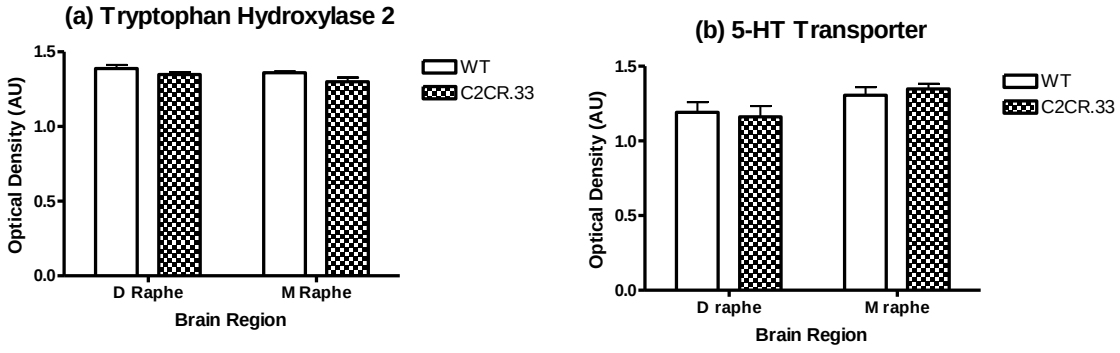


Figure 5.3: Tryptophan hydroxylase 2 and 5-HT transporter in male C2CR.33 mice Compared to WT mice, C2CR.33 mice had reduced TPH2 (a) but no alteration in the 5-HT transporter was found between genotypes (b). All mice were male, backcrossed to C57BL/6 for 7 generations. Expression levels were measured by optical densitometry of *in situ* hybridisation autoradiographs. Data are expressed as mean±SEM; C2CR.33 mice were compared to WT littermate control mice by 2-way ANOVA with Bonferroni posttests; n=5-6 per group.

5.2.4 Effect of 5-HT_{2C} receptor over- or under-expression on brain 5-HT and its metabolites

Compensatory changes in the 5-HT system could occur in levels of 5HT itself, levels of 5-HT metabolites, or in expression of the various 5-HT receptors in response to 5-HT_{2C} receptor over-expression or deficiency. Levels of 5-HT and its main metabolite, 5-hydroxyindoleacetic acid (5-HIAA), were measured in four brain regions (cortex, hippocampus, cerebellum which includes the hindbrain and the remainder of the brain which includes thalamic regions) by high performance liquid chromatography (HPLC).

Compared to WT mice, 5-HT levels were reduced in the hippocampus in C2CR.33 mice, with a significant interaction of the genotype and region (Figure 5.4a; $F_{2,25}$=3.94; p=0.033). In both genotypes, 5-HT levels were higher in the hippocampus compared to the other brain regions tested (effect of region $F_{2,25}$=72.46; p<0.0001). 5-HIAA levels were reduced in the hippocampus in C2CR.33 mice compared to WT mice, with a significant interaction of the genotype and region (Figure 5.4a; $F_{2,25}$=3.28; p=0.05). In both genotypes, 5-HIAA levels were higher in the hippocampus compared to the other brain regions tested (effect of region $F_{2,25}$=93.79; p<0.0001). To estimate 5-HT breakdown the ratio of 5-HT to 5-HIAA was calculated and was unaltered between C2CR.33 and WT mice suggesting that in the hippocampus less 5-HT must be released and broken down (Figure 5.4c; $F_{2,25}$=2.70; p=0.0864).

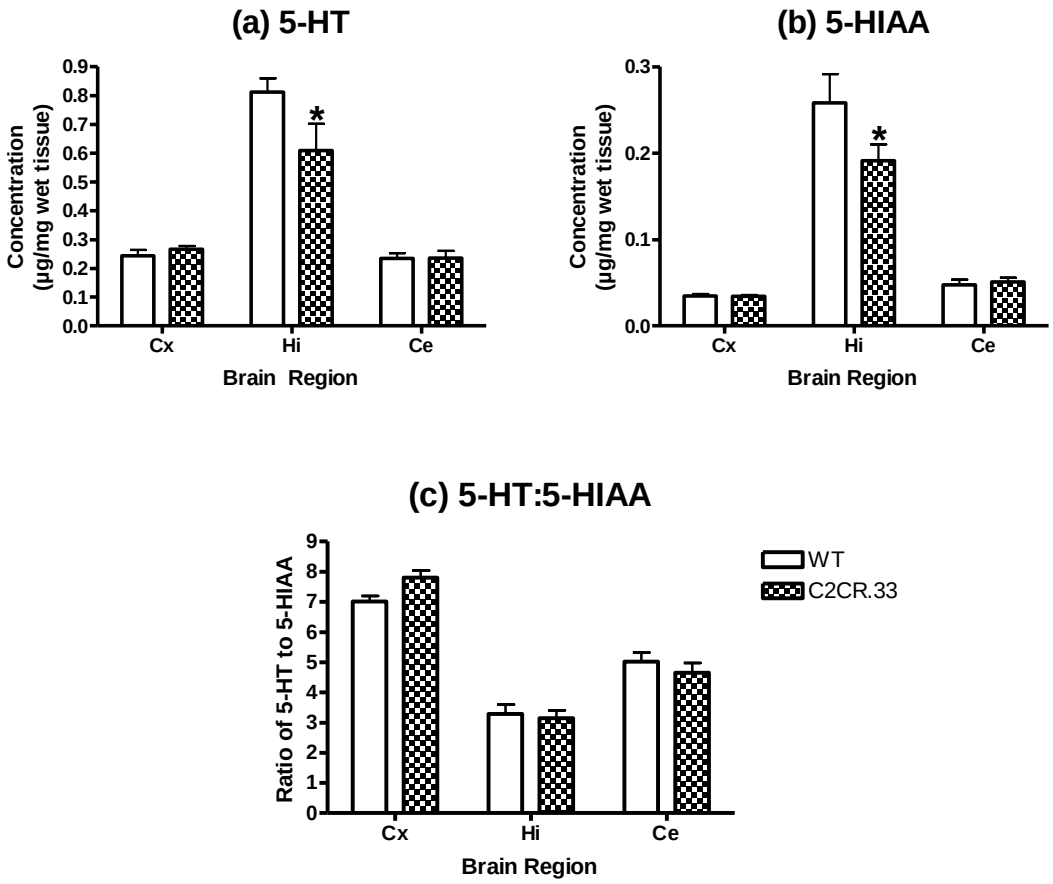


Figure 5.4: Higher performance liquid chromatography (HPLC) in male C2CR.33 mice for 5-HT and its metabolites

5-HT levels were reduced in the hippocampus of C2CR.33 mice (a) compared to WT mice. Compared to WT mice, 5-HIAA levels were reduced in the hippocampus (b) in C2CR.33 mice. The 5-HT to 5-HIAA ratio was unaltered between genotypes (c). High performance liquid chromatography (HPLC) analysis was used to analyse brain regions (Cx = cortex, Hi = hippocampus, Cb = cerebellum and Re = remainder of brain including thalamic and hypothalamus regions) in C2CR.33 mice backcrossed to C57BL/6 for 7 generations. Data are expressed as mean±SEM; C2CR.33 mice were compared to WT littermate control mice by 2-way ANOVA with Bonferroni posttests; n=5-6. * $p<0.05$.

Measurement of 5-HT levels in four brain regions (cortex, hippocampus, cerebellum and the remainder of the brain which includes thalamic regions) showed no difference between TetO-2CR and WT mice in each brain region examined (Figure 5.5a; interaction: $F_{3,32}$=1.18; p=0.334), although levels of 5-HT differed between brain regions ($F_{3,32}$=28.46; p<0.0001) with the hippocampus and the "remainder" (which includes the raphe nucleus) having higher levels than the cortex and cerebellum. 5-hydroxyindoleacetic acid (5-HIAA), the main metabolite of 5-HT, was higher in the hippocampus of TetO-2CR mice than in WT mice (Figure 5.5b) suggesting an increase in 5-HT breakdown and possibly release. Moreover, in both genotypes levels of this metabolite were higher in the hippocampus than in other regions examined with a significant interaction between genotype and region being found (Figure 5.5b interaction: $F_{3,32}$=3.06; p=0.042; genotype: $F_{1,32}$=0.78; p=0.383; region $F_{3,32}$=53.57; p<0.0001). To estimate 5-HT breakdown the ratio of 5-HT to 5-HIAA was calculated and was unaltered between TetO-2CR and WT mice suggesting that in the hippocampus more 5-HT must be released and broken down (Figure 5.5c; $F_{1,32}$=0.54; p=0.467).

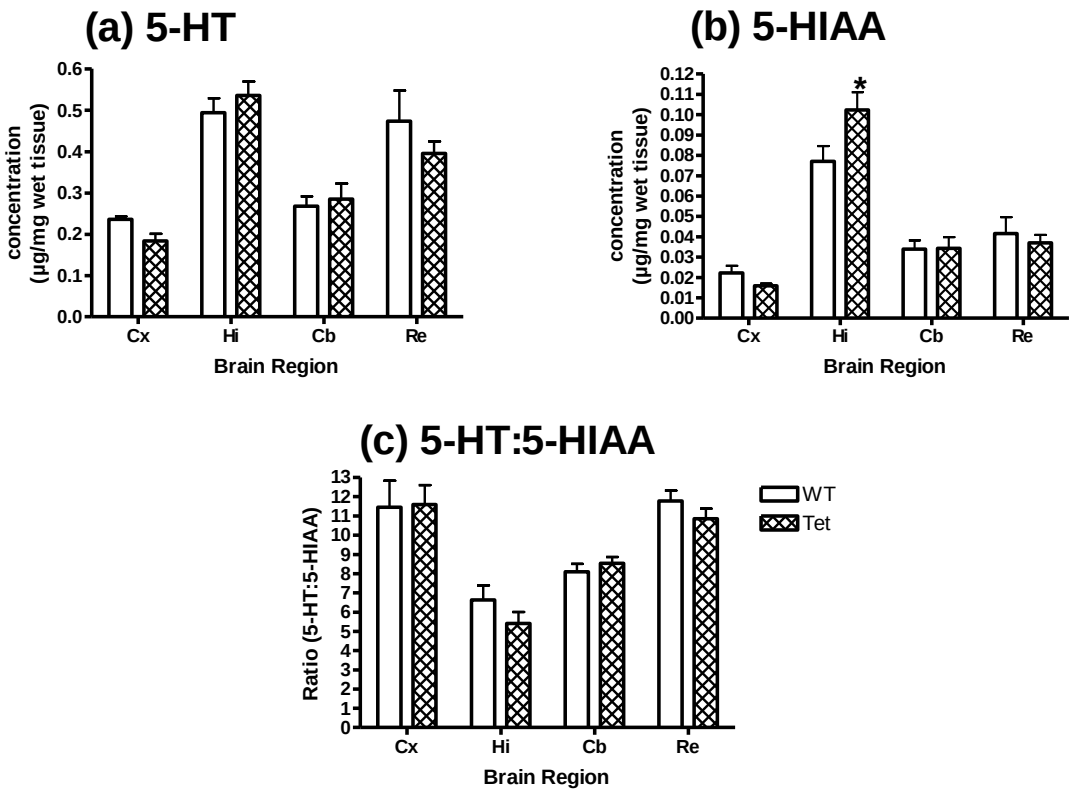


Figure 5.5: High performance liquid chromatography (HPLC) in male TetO-2CR mice for 5-HT and its metabolites

5-HT levels was higher in the hippocampus and "remainder" in both genotypes but no alterations were found between genotypes (a). 5-HIAA is increased in the hippocampus of TetO-2CR mice compared to WT mice (b). 5-HT breakdown was unaltered between genotypes (c). High performance liquid chromatography (HPLC) was used to analyse brain regions (Cx = cortex, Hi = hippocampus, Cb = cerebellum and Re = remainder of brain including thalamic and hypothalamus regions) in TetO-2CR mice, backcrossed to C57BL/6 for 5 generations, 12 months of age. Data are expressed as mean±SEM and analysed by 2-way ANOVA with Bonferroni posttests; n=5 per group. * $p<0.05$.

5.3 The Dopaminergic System:

It has been suggested that the 5-HT_{2C} receptor plays a role in regulating dopamine (DA) release (De Deurwaerdere *et al*., 2004) and so aspects of the DA system were examined to determine if they were affected by altered 5-HT_{2C} receptor levels.

5.3.1 Tyrosine Hydroxylase mRNA levels

Tyrosine hydroxylase (TH) is a rate-limiting enzyme in the production of dopamine and is present in the ventral tegmental area (VTA), the retrorubral fields and the substantia nigra (SN). The substantia nigra (SN) receives dense serotonergic input and 5-HT_{2C} receptors are expressed in the cell bodies of GABA-containing neurones in both the SN pars reticulate and the VTA (Moga & Moore., 1997; Moyer & Kennaway., 1999; Di Matteo *et al*., 2001).

TH mRNA levels were significantly reduced in C2CR.10 mice compared to WT mice (Figure 5.6a; p=0.0109), however no difference was found in C2CR.33 mice (Figure 5.6b; p=0.9426) compared to WT mice. Levels of TH mRNA were similar in the SN and VTA of TetO-2CR mice compared to WT mice (Figure 5.6c; p=0.388) suggesting that the DA production pathway may be unaltered by 5-HT_{2C} receptor deficiency, however, changes in enzyme activity or pathway flux may occur without changes in mRNA levels.

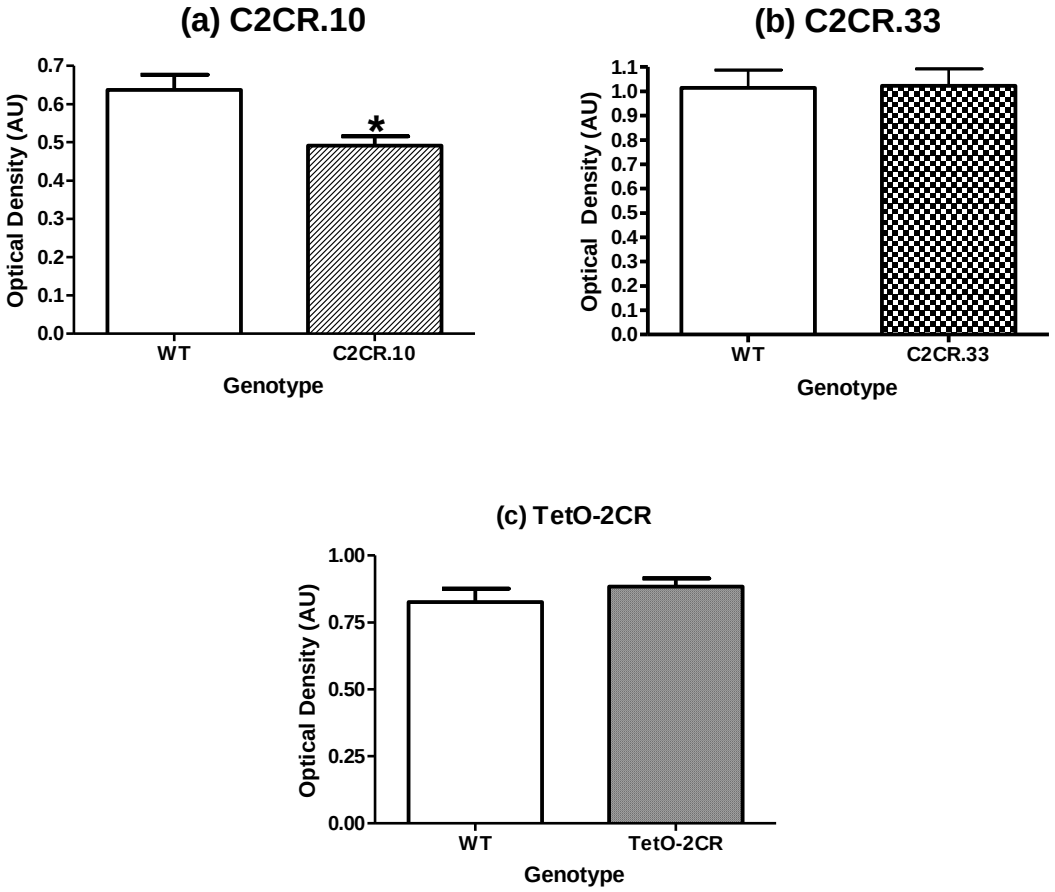


Figure 5.6: Tyrosine Hydroxylase (TH) mRNA levels in substantia nigra/ventral tegmental area

TH mRNA expression is reduced in the SN/VTA in C2CR.10 mice compared to WT mice (a). All mice were males backcrossed to C57BL/6 for 2 generations. Compared to WT mice, TH mRNA expression was unaltered in C2CR.33 mice (b) in the SN/VTA. All mice were males backcrossed to C57Bl6 for 7 generations. TH mRNA expression was unaltered in the SN/VTA (c) of TetO-2CR mice compared to WT littermate control mice. All mice were males, backcrossed to C57BL/6 for 2 generations. Expression levels were measured by optical densitometry of *in situ* hybridisation autoradiographs. Data are expressed as mean±SEM; C2CR.10, C2CR.33 or TetO-2CR mice were compared to WT littermate control mice by unpaired Student's t test; n=5-6. * $p<0.05$.

5.3.2 Dopamine 2 receptor (D2) mRNA levels

The dopamine 2 receptor (D2) is expressed in the caudate putamen (CPu), which is the end point of the nigrostriatal DA pathway (Drago *et al*., 1994). D2 receptor mRNA was unaltered in C2CR.10 mice compared to WT mice (Figure 4.7a; p=0.9373). In C2CR.33 and WT mice, D2 receptor mRNA was increased in the caudate putamen (CPu) compared to other regions tested (Figure 5.7b; $F_{2,24}$=10.00; p=0.0007). D2 receptor mRNA levels did not differ between genotypes (Figure 5.7b; effect of genotype $F_{1,24}$=0.37; p=0.5504; interaction of genotype with region $F_{2,24}$=0.74; p=0.4866

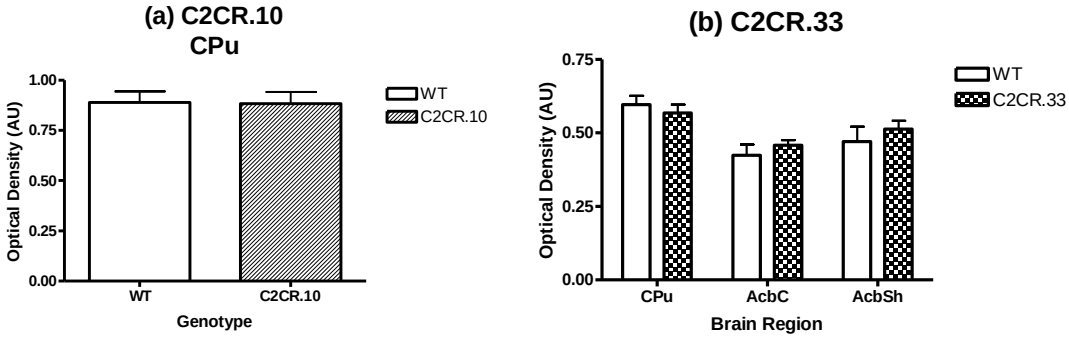


Figure 5.7: Dopamine 2 receptor mRNA expression in male C2CR.10 or C2CR.33 mice Dopamine 2 receptor (D2) is unaltered in the CPu in C2CR.10 mice (a) compared to WT mice. All mice backcrossed to C57BL/6 for 2 generations. In C2CR.33 mice D2 receptor is unaltered (b) compared to WT mice. All mice backcrossed to C57BL/6 for 7 generations. Expression levels were measured by optical densitometry of *in situ* hybridisation autoradiographs. Data are expressed as mean±SEM; C2CR.33 compared to WT littermate control mice by 2-way ANOVA with Bonferroni posttests; n=6 per group.

CPu = caudate putamen; AcbC= nucleus accumbens core; AcbSh=nucleus accumbens shell

5.3.3 DA and its metabolites

DA is catabolised into homovanillic acid (HVA) and noradrenaline (NA) through two independent pathways (see Chapter 1). Levels of DA, HVA and NA were measured in four brain regions (cortex, hippocampus, cerebellum and the 'remainder' of the brain which includes thalamic regions and the basal ganglia) by high performance liquid chromatography (HPLC) with fluorescence detection.

In 5-HT_{2C} receptor over-expressing mice (C2CR.33 mice) DA levels were significantly increased in the cortex, but not other regions, compared to WT mice (Figure 5.8a; interaction of genotype and region $F_{2,25}$=10.99; p=0.0004; effect of genotype $F_{1,25}$=32.50; p<0.0001). The results for HVA in the 'remainder' section were excluded in this group of mice due to large variation. Accordingly for analysis of DA and HVA levels, DA was reanalysed without the 'remainder' section to allow for statistical analysis. In the hippocampus levels of HVA were reduced in C2CR.33 mice compared to WT mice, with a trend of a significant interaction between genotype and region (Figure 5.8b; $F_{2,25}$=2.96; p=0.0701). In both genotypes HVA levels were elevated in the hippocampus compared to other regions (effect of region $F_{2,25}$=24.41; p=<0.0001). The ratio of DA to HVA (indicating DA breakdown) was increased in the cortex of C2CR.33 mice, but did not differ from WT mice in other regions of brain (Figure 5.8c; interaction $F_{2,25}$=10.23; p=0.0006; effect of genotype $F_{1,25}$=11.05; p=0.0027). These results suggest slower breakdown of DA into HVA in the cortex in C2CR.33 mice compared to WT mice.

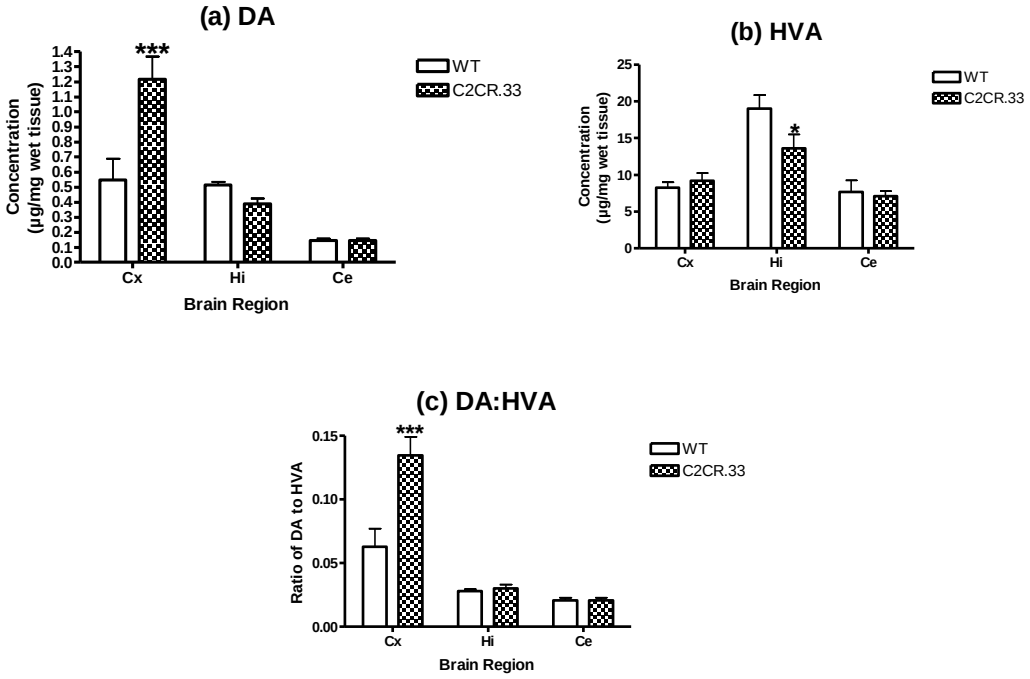


Figure 5.8: High performance liquid chromatography (HPLC) of catecholamines in male C2CR.33 mice

DA levels were increased in the cortex of C2CR.33 mice (a) compared to WT controls. HVA levels were reduced in the hippocampus of C2CR.33 (b) and an increased DA to HVA ratio was found in the cortex of C2CR.33 (c). High performance liquid chromatography (HPLC) analysis was used to analyse brain regions (Cx = cortex, Hi = hippocampus, Cb = cerebellum and Re = remainder of brain including thalamic and hypothalamus regions). All mice were males backcrossed to C57BL/6 for 7 generations. Data are expressed as mean±SEM; C2CR.33 mice compared to WT littermate control mice by 2-way ANOVA with Bonferroni posttests; n=5-6 per group. * $p<0.05$, *** $p<0.001$.

As previously mentioned DA levels were re analysed including the 'remainder' section and DA was still significantly increased in the cortex in C2CR.33 mice compared to WT mice (Figure 5.9a; interaction of genotype with region; $F_{3,32}$=10.09; p<0.0001). There was no difference in NA levels between C2CR.33 and WT mice in any region examined (Figure 5.9b; interaction $F_{3,32}$=1.31; p=0.289 effect of genotype $F_{1,32}$=0.67; 0.42). Levels of NA were higher in the hippocampus of both genotypes compared to the other regions tested ($F_{3,32}$=30.80; p<0.0001). The ratio of DA to NA (indicating production of NA from DA) was increased in the cortex of C2CR.33 mice compared to WT mice (Figure 5.9c; interaction $F_{3,32}$=2.86; p=0.0524) suggesting slower conversion of DA to NA.

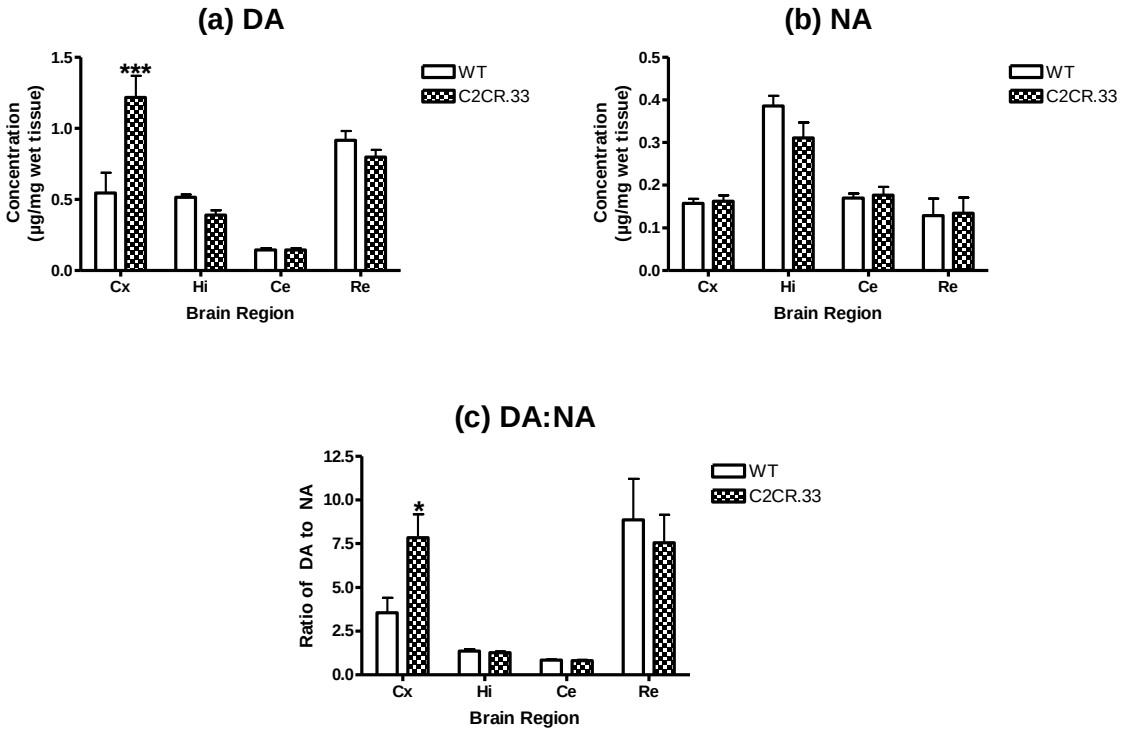


Figure 5.9: High performance liquid chromatography (HPLC) of noradrenaline in male C2CR.33 mice

DA levels were re analysed (a) to incorporate the 'remainder' fraction and the increased DA level in the cortex remained. NA was unaltered between genotypes (b) and the DA to HVA ratio was increased in the cortex of C2CR.33 (c). High performance liquid chromatography (HPLC) analysis was used to analyse brain regions (Cx = cortex, Hi = hippocampus, Cb = cerebellum and Re = remainder of brain including thalamic and hypothalamus regions). All mice were males backcrossed to C57BL/6 for 7 generations. Data are expressed as mean±SEM; C2CR.33 mice compared to WT littermate control mice by 2-way ANOVA with Bonferroni posttests; n=5-6 per group. * p<0.05, *** p<0.001.

In 5-HT_{2C} receptor under-expressing mice (TetO-2CR mice) DA levels did not differ between genotype in any region examined (Figure 5.10a; $F_{1,32}$=0.04; p=0.84), although they were higher in the "remainder" section, which includes the basal ganglia, than in other regions in both genotypes ($F_{3,32}$=28.68; p<0.0001). Homovanillic acid (HVA), a metabolite of DA, was lower in the cortex and "remainder" of TetO-2CR mice than in WT mice (Figure 5.10b; effect of genotype $F_{1,32}$=8.05; p=0.008) although there was no interaction of genotype and region ($F_{3,32}$=1.03; p=0.394). The ratio of DA to HVA (indicating DA breakdown) was increased in the "remainder" fraction of brain from TetO-2CR mice, but did not differ from WT mice in other regions of brain (Figure 5.10c; effect of genotype $F_{1,32}$=5.38; p=0.027) with a significant interaction of the genotype with the region ($F_{3,32}$=2.90; p=0.05). These results suggest slower breakdown of DA into HVA in the cortex and 'remainder' of TetO-2CR mice compared to WT control mice.

Levels of noradrenaline (NA) differed in a genotype and region specific manner with higher levels in the hippocampus and reduced levels in the cortex and "remainder" of TetO-2CR mice compared to WT mice (Figure 5.10d; interaction of genotype and region $F_{3,32}$=3.01; p=0.045). NA is produced from DA, and the ratio of DA to NA was increased in both genotypes in the cortex and remainder compared to other regions ($F_{3,32}$=38.90; p<0.0001) but did not differ between TetO-2CR and WT mice (Figure 5.10e; effect of genotype $F_{1,32}$=2.54; p=0.121; interaction of genotype and region $F_{3,32}$=1.29; p=0.294).

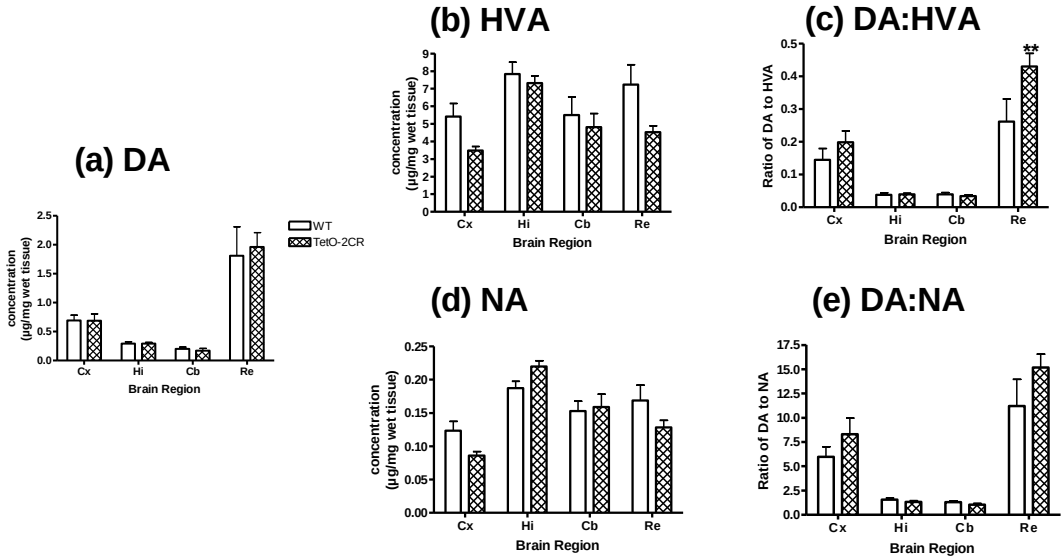


Figure 5.10: High performance liquid chromatography (HPLC) in male TetO-2CR mice for DA, HVA and NA

DA levels were unaltered between genotypes, although levels were higher in both genotypes in the "remainder" (a). HVA was reduced in the cortex and "remainder" of TetO-2CR mice compared to WT mice but no interaction of genotype and region was found (b). The ratio of DA to HVA was increased in the "remainder" of TetO-2CR mice compared to WT mice but was unaltered in other regions (c). NA is increased in the hippocampus and decreased in the cortex and "remainder" of TetO-2CR mice compared to WT mice (d). The ratio of DA to NA was increased in both genotypes in the cortex and remainder compared to other regions but no alterations were found between gentoypes (e). High process liquid chromatography (HPLC) was used to analyse brain regions (Cx = cortex, Hi = hippocampus, Cb = cerebellum and Re = "remainder" of brain including thalamic and hypothalamus regions). All mice were males, backcrossed to C57BL/6 for 5 generations, 12 months of age. Data are expressed as mean±SEM, TetO-2CR mice compared to WT littermate control mice by 2-way ANOVA with Bonferroni posttests; n=5 per group. ** $p<0.01$.

5.4 Discussion

It was established in Chapter 3 (Section 3.2.2) that C2CR.10 mice over-express the 5-HT_{2C} receptor in a region-specific manner while C2CR.33 mice had widespread over-expression in the forebrain. While, TetO-2CR mice under-express the 5-HT_{2C} receptor throughout the brain (see Chapter 4 section 4.2.2). Because the 5-HT_{2C} receptor is either over-expressed or deficient throughout development, normal development may be perturbed or other receptors/ pathways may have been altered during development to compensate for the increased or lack of signalling through the 5-HT_{2C} receptor. Alterations in aspects of the central 5-HT system functioning such as TPH2 activity, 5-HT levels, MAO-A activity and 5-HT_{1A} receptor levels are associated with altered aggression levels (Brown *et al*., 1979; Gibbons *et al*., 1979; Popova *et* al., 1991; Cases *et al*., 1995; Kulikov *et al*., 2005; Popova., 2008; Vage *et al*., 2010).

5.4.1 5-HT_{1A} receptor mRNA expression

Down-regulation of 5-HT_{1A} receptors is associated with depression and anxiety (Gross *et al*., 2000) and disruption of the 5-HT_{1A} receptor gene increases anxiety-like behaviours (Parks *et* al., 1998; Ramboz *et al*., 1998; Gingrich *et al*., 2000; Gingrich & Hen., 2001; Olivier *et al*., 2001) with transgenic re-expression of 5-HT_{1A} receptor in the amygdala and hippocampus being sufficient to rescue this behavioural phenotype (Gross *et al*., 2002). Motor coordination is not altered in 5-HT_{1A} receptor knock-out (KO) mice (Heisler *et al*., 1998). In 5-HT_{2C} receptor KO mice, no alterations were found in 5-HT receptor subtypes (5-HT 1A, 1B/1D, 1F, 2A, 2B, 4 and 7; Lopez-Gimenez *et al*., 2002).

However, compared to WT mice, C2CR.10 mice had reduced 5-HT_{1A} receptor mRNA levels in the CA1 field of the dorsal hippocampus in response to increased 5-

HT_{2C} receptors in all regions of the dorsal hippocampus. This is consistent with C2CR.33 mice which had reduced levels of 5-HT_{1A} receptor in all regions of the dorsal hippocampus in response to a much greater increase in 5-HT_{2C} receptor mRNA in these regions. No differences in 5-HT_{2C} receptor or 5-HT_{1A} receptor mRNA levels were found in the amygdala in C2CR.10 mice however C2CR.33 mice had increased and decreased mRNA of these receptors respectively. Both an elevation in 5-HT_{2C} receptor and reduction in 5-HT_{1A} receptor signalling increase anxiety-like behaviour, hence this compensatory decrease in 5-HT_{1A} receptor mRNA levels was not the cause of the normalisation in anxiety levels in C2CR.33 mice. In the TetO-2CR mice, 5-HT_{1A} receptor mRNA levels were elevated in the CA1 field of the dorsal hippocampus and in the amygdala. This elevation in 5-HT_{1A} receptor levels would be predicted to have a similar effect on anxiety-like behaviour to that hypothesised to results from the reduction in the 5-HT_{2C} receptor. These results demonstrate cross talk between the 5-HT_{2C} receptor and 5-HT_{1A} receptor.

In the raphe nucleus somatodendritic 5-HT_{1A} receptors act as autoreceptors causing a reduction in 5-HT synthesis and release into post synaptic sites (Blier *et al*., 1998; Richardson-Jones *et al*., 2010). In the dorsal and median raphe nucleus 5-HT_{2C} receptor mRNA levels were unaltered in C2CR.33 mice and levels of 5-HT_{1A} receptors were also unaltered between genotypes in this region. This suggests that there was no alteration in regulation of 5-HT synthesis by 5-HT_{1A} autoreceptors.

5.4.2 5-HT_{2A} receptor mRNA expression

The structures where 5-HT_{2A} receptors are expressed modulate behaviours associated with anxiety state (Millan 2003). Global disruption of 5-HT_{2A} receptor signalling in mice reduces anxiety-like behaviour but does not affect depressive-like or fear-conditioned behaviour (Weisstaub *et al*., 2006). Selective restoration of 5-HT_{2A} receptors in the cortex of these KO mice normalises conflict anxiety behaviours, suggesting a specific role for cortical 5-HT_{2A} receptor function.

Compared to WT mice, 5-HT_{2A} receptor mRNA was unaltered in the ventral hippocampus of C2CR10 mice, however no increase in 5-HT_{2C} receptor mRNA was found in the CA1 or CA2 fields of the ventral hippocampus. Similarly, 5-HT_{2C} receptor deficient mice (TetO-2CR) had unaltered 5-HT_{2A} receptor mRNA levels in the ventral hippocampus. In C2CR.33 mice, 5-HT_{2C} receptor mRNA levels were high in the ventral hippocampus and 5-HT_{2A} receptor mRNA was found to be decreased in the CA1 of the ventral hippocampus. Both an elevation in 5-HT_{2C} receptor and reduction in 5-HT_{2A} receptor signalling increase anxiety-like behaviour, hence this compensatory decrease in 5-HT_{2A} receptor mRNA levels would not have normalised anxiety levels in C2CR.33 mice.

5.4.3 Other aspects of the 5-HT system

Importantly, perturbation of the 5-HT system by disruption of the gene encoding the 5-HT transporter has region-specific compensatory alterations in 5-HT receptors. 5-HT_{1A} receptors were decreased in the dorsal raphe and increased in the hippocampus (Fabre *et al.*, 2000; Li *et al.*, 2000), 5-HT_{1B} receptors were decreased in the substantia nigra (Fabre *et al.*, 2000) and 5-HT_{2A} receptors were decreased in the claustrum, cerebral cortex and lateral striatum (Rioux *et* al., 1999) of 5-HTT KO mice. The density of 5-HT_{2A} receptors (increased in hypothalamus and septum but reduced in striatum) and 5-HT_{2C} receptors (increased in amygdala and choroid plexus) are changed in a region-specific manner in 5-HTT KO mice (Li *et al.*, 2003). 5-HTT mRNA levels did not differ between C2CR.33 and WT mice in the raphe nucleus and It would be interesting to examine levels of 5-HTT in TetO-2CR mice to determine if transportation of 5-HT may be altered.

Tryptophan hydroxylase 2 (TPH2) is the rate-limiting enzyme in the brain in 5-HT production. A single nucleotide polymorphism at the *mTPH2* locus (C1473G) reduced 5-HT synthesis by 55% when expressed in PC-12 cells (Zhang *et al.*, 2004)

and in mice this polymorphism determines differences in 5-HT synthesis rates but not tissue content in C57BL/6 mice (Siesser *et al*., 2010). Female *Tph2* KO mice have increased aggressive behaviour (Alenina *et al*., 2009) and female aggression is directly linked to anxiety and depressive disorders (Bosch *et al*., 2005). In the raphe nucleus in C2CR.33 mice TPH2 mRNA levels were reduced indicating less 5-HT may be produced.

Another area to consider is if 5-HT levels and its main metabolite, 5-HIAA, have been affected and could potentially be normalising any anxiety-like or depressive-like behaviour. Alterations in 5-HT levels play a role in mood with selective serotonin reuptake inhibitors (SSRIs) used as a treatment for major depression (Deshauer *et al*., 2008).

5-HT levels and 5-HIAA (the main metabolite of 5-HT) were decreased in the hippocampus, but no other brain region, in C2CR.33 mice compared to WT mice. Although no alterations in the levels of 5-HT or the conversion rate of 5-HT to 5-HIAA were found, 5-HIAA was elevated in the hippocampus of TetO-2CR compared to WT mice. These results suggest that less 5-HT is being produced for release in the hippocampus in mice that over-express 5-HT_{2C} receptors, while in mice that under-express 5-HT_{2C} receptors more 5-HT must be released and broken down in the hippocampus.

5.4.4 5-HT_{2C} receptor expression levels influence the mesocorticolimbic DA pathway

5-HT is believed to exert an inhibitory control over both the mesolimbic and nigrostriatal DA systems through the 5-HT_{2C} receptor, with antagonists of this receptor increasing DA release in the NA and prefrontal cortex by increasing the

firing rate and bursting activity of DA-containing neurons in the VTA (Di Matteo et al 2001; McMahon et al 2001; Di Matteo et al 2002; Higgins & Fletcher 2003; De Deurwaerdere et al 2004). It was therefore hypothesised that altered 5-HT_{2C} receptor expression levels may have caused alterations in the DA system. Tyrosine hydroxylase (TH) is the rate-limiting enzyme in DA production in the VTA. In C2CR.10 mice, 5-HT_{2C} receptor mRNA levels were increased in the SN/VTA brain region (Chapter 3 section 3.2.2) and interestingly TH mRNA levels were reduced in this region. Therefore, it was hypothesised that DA production may be reduced in C2CR.10 mice. Unfortunately due restraints HPLC analysis of DA levels and its metabolites was not carried out in this mouse line. In C2CR.33 mice, 5-HT_{2C} receptor mRNA did not differ from WT mice in the SN/VTA, nor did TH mRNA levels.

There are two major DA pathways; the mesocortcolimbic pathway and the nigrostriatal pathway. The mesocorticolimbic pathway projects from the VTA to cortical and limbic structures, including the nucleus accumbens and is altered in 5-HT_{2C} receptor KO mice, with increased DA levels in the nucleus accumbens (Abdallah *et al*., 2009). Loss of DA signalling in the nucleus accumbens is proposed to cause decreased activity of dopaminergic reward mechanisms, which underlie feelings of anhedonia. DA levels did not differ in TetO-2CR mice compared to WT mice. In response to 5-HT_{2C} receptor over-expression in C2CR.33 mice DA levels were increased and HVA levels were decreased in the cortex. To estimate DA release and breakdown the ratio of DA to HVA was used and it was concluded that DA release was inhibited by increased 5-HT_{2C} receptors in the cortex, where 5-HT_{2C} receptor is not usually present (Chapter 3 section 3.2.2). This suggests that the mesocorticolimbic pathway is more sensitive to increased or decreased levels of 5-HT_{2C} receptor signalling. D1 receptor is present in the hippocampus and prefrontal cortex but due to problems with the riboprobe these *in situ* hybridisation experiments failed.

The nigrostriatal DA pathway is involved in locomotion. Due to the strong hypolocomotor phenotype and reduced TH mRNA levels in the SN/VTA in C2CR.10 mice (from generation 2 mice; Chapter 3 section 3.2.5 and Chapter 4 section 4.3.5) it was decided to examine both D1 and D2 receptors in the dorsal caudate putamen (CPu), which is part of the nigrostriatal system. D1 and D2 receptors are present on different populations of spiny projection neurones in the dorsal CPu. The direct pathway projects from the CPu to the substantia nigra and entopeduncular nucleus (the output nuclei of the basal ganglia) and the neurons that give rise to these projections have high levels of D1 receptor and the neuropeptides substance P and dynorphin. The indirect pathway projects from the CPu to the globus pallidus and the basal ganglia output nuclei indirectly and the neurons that give rise to this projection express high levels of D2 receptor and enkephalin (Drago et al 1994). It is unlikely that alterations are present in the indirect pathway projecting from the nigrostriatal system as D2 receptor mRNA levels were unaltered in the dorsal CPu of C2CR.10 mice. This was also the case in C2CR.33 mice providing further evidence for the hypothesis that it is the mesocorticolimbic pathway that is altered in response to 5-HT_{2C} receptor signalling.

Alterations in the DA system could contribute to the hyperlocomotion found in TetO-2CR mice in the wheel running cages (see Chapter 4). It has been reported that under basal conditions 5-HT_{2C} receptor KO mice show no difference in DA levels in the nucleus accumbens or dorsal striatum (Rocha *et al.*, 2002). However, more recently, increased basal DA levels were shown in the nucleus accumbens of 5-HT_{2C} receptor KO mice (Abdallah *et al.*, 2009). No alterations in DA levels or TH, the rate limiting enzyme in DA production, were found in TetO-2CR mice compared to WT under basal conditions indicating that hyperlocomotion is not due to reduced inhibition on DA release as a consequence of 5-HT_{2C} receptor deficiency in TetO-2CR mice.

Other aspects of the DA system could also be altered to compensate for 5-HT_{2C} receptor deficiency in TetO-2CR mice which could alter locomotion or depressive behaviours. Levels of HVA, a major DA metabolite, are altered in the plasma of schizophrenic patients (Arrúe *et al*., 2010). HVA was lower in the brain of TetO-2CR mice and the ratio of DA to HVA was found to be increased in the remainder brain section (which contains the substantia nigra) in TetO-2CR mice indicating that breakdown of DA to HVA may be slower. Hence in future studies it would be important to measure monoamine oxidase and catechol-O-methyl transferase as they are involved in DA breakdown and may not be functioning optimally when there is a deficiency in the 5-HT_{2C} receptor.

DA is also the precursor to noradrenaline (NA) which is involved in depression, influences the reward system and is involved in attention deficit/ hyperactivity disorder (Perona *et al*., 2008; Dell'Osso *et al*., 2010; Lim *et al*., 2010). In addition to its role in regulation of DA release, the 5-HT_{2C} receptor agonist, RO 60-0175, suppresses frontal cortex NA levels in rats while the 5-HT_{2C} receptor antagonist, SB-242084, increases NA levels. (Millan *et al*., 1998). Serotonin and noradrenaline reuptake inhibitors (SNRIs) are used in the treatment of some anxiety disorders. Noradrenaline levels were unaltered in C2CR.33 mice and the ratio of DA to noradrenaline was increased in the cortex. These results taken in combination with those from the HVA pathway suggest that 5-HT_{2C} receptor over-expression has caused less DA to be released in the cortex and, in order to maintain normal noradrenaline levels, the balance of the two DA catabolic pathways is altered in favour of noradrenaline production therefore causing a decrease in HVA levels.

The ratio of DA to NA, indicating possible conversion rate, was unaffected in TetO-2CR mice but interestingly NA levels were increased in the hippocampus. 5-HT and noradrenaline reuptake inhibitors (SNRIs) are used in the treatment of some anxiety disorders and so increased concentration of NA at the synapse is predicted to have a beneficial effect on mood, including recovery of motivation (Dell'Osso *et al*., 2010).

It would therefore be interesting to examine NA concentrations in the synapse of TetO-2CR mice which due to time constraints was not undertaken. One method to measure NA levels would be to use dual-probe microdialysis.

Dihydroxyphenylacetic acid (DOPAC) is produced from DA after reuptake by the nerve terminal and so quantitation of DOPAC concentration would have given a more accurate idea of DA neurotransmission. Another limitation to these results is the exclusion of an analysis of dihydroxyphenylacetic acid (DOPAC) concentration which was unable to be carried out due to a shortage in machine availability.

5.4.5 Concluding Remarks

These results suggest that over-expression of the 5-HT_{2C} receptor in the hippocampus in C2CR.33 mice caused compensatory alterations which reduced 5-HT_{1A} receptor (also reduced in amygdala) and 5-HT_{2A} receptor signalling. Region-specific 5-HT_{2C} receptor over-expression in C2CR.10 mice caused region-specific 5-HT_{1A} receptor reduction while TetO-2CR mice had increased 5-HT_{1A} receptor expression. This suggests influential cross talk between 5-HT_{2C} receptors and 5-HT_{1A} receptors. 5-HT production (indicated by reduced TPH2) and release in the hippocampus was reduced in C2CR.33 mice but increased in TetO-2CR mice. Over-expression of 5-HT_{2C} receptors influences the DA system with reduced TH levels in response to increased 5-HT_{2C} receptor in the SN/VTA in C2CR.10 mice. DA release in the cortex was inhibited by increased 5-HT_{2C} receptor levels in the cortex in C2CR.33 mice. The hyperactive phenotype in TetO-2CR mice does not appear to be due to decreased inhibition of the DA system. It is hypothesised that the mesocorticolimbic DA pathway is more sensitive to 5-HT_{2C} receptor signalling alterations than the nigrostriatal DA pathway.

CHAPTER 6:

DISCUSSION

Behavioural phenotypes caused by alterations in the 5-HT_{2C} receptor appear to be sensitive to either the genetic background of the mice, environmental changes and/or alterations in other receptors to compensate for differences in 5-HT_{2C} receptor signalling. This is suggested due to several reasons, the first being that C2CR.10 and C2CR.33 mice, which over-express the 5-HT_{2C} receptor, originally had an anxiogenic and hypolocomotor phenotype which was lost in later generations (Chapter 3). Secondly TetO-2CR mice, which under-express the 5-HT_{2C} receptor, originally had a hyperlocomotor phenotype which was lost for several generations before returning (Chapter 4). In order to hypothesise a cause for the alterations in phenotype several areas had to be considered. Sensitivity to environment could account for the difference in results in these 5-HT_{2C} over-expressing and under-expressing models as a move in animal facilities was carried out, afterwhich the behavioural phenotype was no longer found. Another possibility was the genetic background of the mouse lines. All three lines originated on a mixed genetic background, which differed between the over-expressing and the under-expressing 5-HT_{2C} receptor mice, and following backcrossing towards a congenic C57BL/6 genetic background phenotypes were lost in all lines. Interestingly 5-HT_{2C} receptor KO mice had an anxiolytic phenotype (Heisler *et al*., 2007b) which was not found more recently (Hill *et al*., 2010).

Alterations in other aspects of the 5-HT system or should also be investigated more thoroughly to compensate for altered 5-HT_{2C} receptor levels in C2CR.10, C2CR.33 and TetO-2CR mice. An inverse relationship was found to exist between the 5-HT_{2C} receptor and 5-HT_{1A} receptor levels. 5-HT levels are also suggested to be increased in response to 5-HT_{2C} receptor deficiency in TetO-2CR mice or decreased in response to 5-HT_{2C} receptor over-expression in C2CR.33 mice. In 5-HT_{2C} receptor KO mice BDNF levels were found to be elevated (Hill *et al*., 2010) and so BDNF levels should also be examined in C2CR.10, C2CR.33 and TetO-2CR mice.

The extent of editing of the 5-HT_{2C} receptor is another area to consider when examining the interaction of the 5-HT_{2C} receptor with other systems and the resulting behavioural phenotype. The 5-HT_{2C} receptor undergoes A to I editing which alters the G-protein coupling of the receptor, with the fully unedited form reported to be constitutively active and edited forms requiring more ligand in order to be activated (Burns *et al*., 1997, Fitzgerald *et* al., 1999, Niswender *et al*., 1999; Berg et al., 2008). Editing levels of 5-HT_{2C} receptor have been found to be increased in depression (Dunlop *et* al., 2006) with elevated editing levels being found in depressed suicide victims (Gurevich *et al*., 2002; Iwamoto *et al*., 2003). Stress increases editing to isoforms which have reduced ligand sensitivity (Englander *et al*., 2005) and early life stress may permanently set this shift in editing levels (Bhansali *et* al., 2007). In a study on 5-HT_{2C} receptor KO mice it was suggested that mis-timed and higher leptin levels during early life in 5-HT_{2C} receptor KO mice (increased leptin levels found at 16, 18 and 20 days old) could programme a change in both feeding and stress neurocircuits (Akana., 2008). It would be interesting to carry out an examination of the extent of editing in TetO-2CR mice as 5-HT_{2C} receptors may be fully unedited to compensate for the reduced numbers. Editing could also be affected by the deficiency in 5-HT_{2C} receptor if TetO-2CR mice had altered levels of leptin (as seen in the 5-HT_{2C} receptor KO mice) which could change stress neurocircuits and therefore editing. The over-expressed 5-HT_{2C} receptors in C2CR.10 and C2CR.33 mice are fully unedited which means that the ratio of unedited to edited 5-HT_{2C} receptor isoforms may be altered. The endogenous 5-HT_{2C} receptors may be fully edited in order to compensate for constitutive activity from the increased unedited isoforms or the number of receptors available on the cell surface may be altered. This is a further area that requires investigation in these lines.

The 5-HT_{2C} receptor has previously been proposed to influence both the nigrostriatal and mesolimbic DA systems (Di Matteo et al 2002; Higgins & Fletcher 2003; De Deurwaerdere et al 2004). Recently, increased DA levels were found in the nucleus accumbens of 5-HT_{2C} receptor KO mice (Abdallah *et al*., 2009) suggesting the mesocorticolimbic DA pathway was altered in these mice. In both C2CR.33 and

TetO-2CR mice aspects of the mesocorticolimbic DA system were altered. Therefore, it is hypothesised that the 5-HT_{2C} receptor plays a more prominent role in regulating the mesocorticolimbic DA system opposed to the nigrostriatal DA system.

Further studies are required to investigate the extent of editing as well as a more thorough investigation of the DA system in C2CR.10, C2CR.33 and TetO-2CR mice. However, these models have provided evidence of compensation in both the 5-HT and DA systems and are novel models to further elucidate the role of the 5-HT_{2C} receptor.

CHAPTER 7:

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